UNIVERSITY OF LONDON THESIS

Degree MD Year 2006 Name of Author OZTURK

Ozkan

CLINICAL ASPECTS OF EMBRYO IMPLANTATION
From the Perspective of Tissue Perfusion

OZKAN OZTURK, MBA, MRCOG

**A thesis submitted to the University of London
for the degree of Doctor of Medicine**

**Academic Department of Obstetrics and Gynaecology
University College London**
2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Hypothesis: Pelvic perfusion is the pivotal factor for the outcome of *in vitro* fertilisation (IVF) treatment once clinical and embryological variables are controlled for their effect.

Demonstration of Hypothesis: In a series of three studies, the clinical aspects of embryo implantation were examined from the perspective of tissue perfusion.

Epidemiological Study: Clinical and embryological data were evaluated to predict multiplicity of implantation and ongoing pregnancy in IVF treatment. Oocyte and embryo quality were appraised and the impact of the number of embryos transferred was assessed.

Study on In-vivo Vascular Physiology: The prognostic role of utero-ovarian perfusion and its pharmacological manipulation with low dose aspirin was evaluated in the outcome of IVF treatment. Serum, follicular fluid vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) concentrations were correlated with Doppler indices.

Study on In-vivo Endometrial Physiology: Endometrial receptivity (in terms of endometrial thickness and echo-pattern) and VEGF-VEGFR concentrations were evaluated with regards to the outcome of frozen-thawed embryo replacement (FTER) during natural and hormone replacement cycles.

Results: The epidemiological study showed that the outcome of IVF treatment was closely associated with the severity of subfertility. Ovarian reserve and response to stimulation were the key factors. The probability of pregnancy was affected by the number and quality of oocytes and by their fertilisation rate and the cleavage rate of the resulting embryos. The potential to provide mature oocytes and high quality embryos was an inherent characteristic of the ovaries and independent of

stimulation protocols. When embryo quality was taken into consideration, the number of embryos transferred no longer affected the chance of pregnancy.

The clinical study showed that the chance of pregnancy was directly dependant upon tissue perfusion. Pregnancy rates were very low with uterine artery pulsatility index >3 (PI) and peri-follicular PI >1. Better ovarian reserve, response to stimulation, endometrial development, implantation and pregnancy rates were associated with low follicular fluid VEGF-VEGFR levels and this was also associated with good uterine and endometrial perfusion. Aspirin (150 mg/day) had no beneficial effect on Doppler indices, ovarian response to stimulation, implantation or pregnancy rates.

Pregnancy rates were similar with naturally and hormonally prepared endometrium in frozen-thawed embryo replacement cycles. Higher serum VEGF and lower VEGFR levels were observed in pregnant cycles, but the differences were not significant. Endometrial echo-pattern and thickness did not affect conception.

Conclusions: Tissue perfusion plays a key role in the physiological steps leading to conception and implantation. Aspirin (150 mg/day) improved neither tissue perfusion nor the outcome of fresh embryo transfer. The type of endometrial preparation did not affect the outcome of frozen-thawed embryo replacement cycles.

Table of Contents

Abbreviations

ACA	Anticardiolipin antibody
Anti-β_2-GPI	Anti-beta$_2$-glycoprotein I antibody
APA	Antiphospholipid antibody
ART	Assisted Reproductive Technology
BMI	Body mass index
CI	Confidence interval
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
CPR	Clinical pregnancy rate
DRRC	Down-regulated replacement cycle
E_2	Oestradiol
ELISA	Enzyme-linked immunosorbent assay
ESR	Embryo survival rate
ET	Embryo transfer
FDA	Food and Drug Administration
FF	Follicular fluid
FIVNAT	French National Register on IVF
Flt 1	Fms-like-tyrosine kinase
FSH	Follicle stimulating hormone
FTER	Frozen-thawed embryo replacement
G	Grade
GnRH	Gonadotrophin releasing hormone
GnRH-a	Gonadotrophin releasing hormone analogue
HCG	Human chorionic gonadotrophin
HFEA	Human Fertilisation and Embryology Authority
HMG	Human menopausal gonadotrophin
HRT	Hormone replacement therapy
ICSI	Intracytoplasmic sperm injection
IL-3	Interleukin 3

IM	Intramuscular
IR	Implantation rate
IU	International unit
IV IG	Intravenous immunoglobulin
IVF	*In vitro* fertilisation
KDR	Kinase domain region
L	Litre
LA	Lupus anticoagulant
LDA	Low dose aspirin
LH	Luteinising hormone
Min	Minute
MLP	Mid-luteal protocol
N	Number of cases
N/A	Not available
NC	Natural cycle
Nmol	Nanomole
NO	Nitric oxide
NRP	Neuropilin
NSAID	Nonsteroidal anti-inflammatory drug
NTG	Nitro-glycerine
NS	Not significant
O_2	Oxygen
OD	Oocyte donation
OHSS	Ovarian hyper-stimulation syndrome
OPR	Ongoing pregnancy rate
OR	Odds ratio
ORS	Ovarian response to stimulation
OS	Ovarian stimulation
P	Significance value
P_4	Progesterone
PCOS	Polycystic ovarian syndrome

PgI_2	Prostacyclin
PI	Pulsatility index
PN	Pro-nuclear
PSV	Peak systolic velocity
PR	Pregnancy rate
PV	Per-vaginal, vaginally, intra-vaginal
RI	Resistance index
ROC	Receiver operating characteristic
S	Stage
S/D	Systolic/Diastolic
SC	Subcutaneous injection
SD	Standard deviation
SE	Standard error
Sec	Second
Sig	Significance
sVEGFR-1	Soluble vascular endothelial growth factor receptor
TAMV	Time averaged maximum velocity
TVS	Transvaginal ultrasonography
TxA_2	Thromboxane A_2
USG	Ultrasonography
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vmax	Maximum peak velocity
Vmin	Minimum diastolic velocity
VPF	Vascular permeability factor
Vs	Versus

Statement of Originality

The studies included in this thesis were conducted in the Academic Departments of Obstetrics and Gynaecology, at University of Aberdeen and University College London. The candidate was the major investigator, responsible for the planning and implementation of the studies and for sample collection and storage. VEGF and VEGFR assays were performed by Ms Jeanette Judah, Department of Women's Health, St Thomas' Hospital. Statistical analysis of the first study was performed by the candidate on the database of the Lister Fertility Clinic.

Acknowledgements

I am indebted to Professor A Templeton, who inspired, encouraged and supervised this research; Dr M Hamilton, for his expert advice in all stages of the clinical trials, and organisation of the funding of the placebo and aspirin tablets; Dr S Bhattacharya, for assisting with the study design; Ms F MacGregor, for assisting with sample preparation and storage; Dr S Abdalla, for allowing the use of his database for the statistical analysis; Professor C Rodeck, who has guided my career, made it possible for me to continue my research at UCL and has read the thesis; Professor E Jauniaux, who has supported me throughout my career in the UK, allowing me to join his research programmes, supervising my work, and organising and funding the VEGF-VEGFR assays; Professor L Poston, for allowing the use of the facilities and expertise in her department; Ms J. Judah, for performing the VEGF-VEGFR assays; Dr M Davies, who read the thesis and Dr E Saridogan, who supervised my work, read this thesis in very short time and provided invaluable guidance - without his help and support, completion of the presented thesis would not have been possible.

The candidate wishes to acknowledge the invaluable contributions of Sister E Graham, Ms L Bell, Ms I Forbes, Ms M Laing, Sister H McBain, Sister E McTavish, Sister S Muray, Dr J Parikh, Sister G Payne, Sister V Peddie, Mr A Srikantharajah and Sister L Stewart.

The candidate further wishes to acknowledge the contribution of Ms P Thomas, for her invaluable linguistic advice and editing of the thesis manuscript.

Publications Directly Related to the Thesis

Ozturk, O., Saridogan, E. and Jauniaux, E. (2004) Drug intervention in early pregnancy after assisted reproductive technology. Reprod Biomed Online., 4, 452-465.

Ozturk, O., Bhattacharya, S., Saridogan, E., Jauniaux, E. and Templeton, A. (2004) Role of utero-ovarian vascular impedance: predictor of ongoing pregnancy in an IVF-embryo transfer programme. Reprod Biomed Online., 3, 299-305.

Ozturk, O., Greaves, M. and Templeton, A. (2002) Aspirin dilemma. Remodelling the hypothesis from a fertility perspective. Hum Reprod., 5, 1146-1148.

Publications Related to the Thesis

Pandian, Z., Bhattacharya, S., Ozturk, O., Serour, G. and Templeton, A. (2004) Number of embryos for transfer following *in vitro* fertilisation or intra-cytoplasmic sperm injection. Cochrane Database Syst Rev., 4, CD003416.

Ozturk, O. and Templeton, (2002) A. *In vitro* fertilisation and risk of multiple pregnancy. Lancet, 9302, 232.

Ozturk, O., Bhattacharya, S., Serour, G. and Templeton, A. (2002) Number of embryos for embryo transfer following *in vitro* fertilisation or intracytoplasmic sperm injection treatment. Protocol for a Cochrane Review. In: The Cochrane Library, Issue 1, 2002. Oxford: Update Software.

Ozturk, O., Bhattacharya, S. and Templeton, A. (2001) Avoiding multiple pregnancies in ART: Evaluation and implementation of new strategies. Hum Reprod., 16, 1319-1321.

Chapters In Books

Ozturk O, Templeton A. Multiple Pregnancies in Assisted Conception. WHO Technical Series, WHO, 2001.

Invited Lectures

Factors affecting the outcome of IVF cycles, 1st Annual Meeting, UCL EGA Institute for Women's Health, 2 December 2005, London

IVF pregnancies and drug interventions, 6th International Symposium on Preimplantation, 19-21 May 2005, London

Oral and Poster Presentations

Role of tissue perfusion in fertility treatment. Poster presentation, 30th British Congress of Obstetrics and Gynaecology, 7 July 2004

Predictive role of utero-ovarian vascular impedance in assisted conception. Poster presentation, Registrar's Prize Meeting of the Royal Society of Medicine. 16.2.2001

Ozturk O, Bhattacharya S, Hamilton M, Templeton A. Predictive role of Utero-ovarian vascular impedance in assisted conception. Abstract, 17th Annual Meeting of ESHRE, Lausanne, 2001.

Low dose aspirin co-treatment in IVF protocols. Oral presentation, North-eastern Obstetrical and Gynaecological Society Annual Meeting. 2.6.2000.

Clinical and embryological factors with prognostic significance in IVF treatment. Poster presentation, North-eastern Obstetrical and Gynaecological Society Annual Meeting. 2.6.2000.

Notes on the Text

This thesis was typed on an IBM PS/2 computer using Microsoft® Office Word 2003. The font used is Arial. The text was printed by a Hewlet-Packard Laserjet 1200 printer. The United Kingdom English Spell-checker facility of Microsoft® Office Word 2003 was used. In the references the spelling used corresponds to that of the original article.

A number of tables comprising data supplementary to the main results are presented on the CD Rom in order to keep the length of the thesis in accordance with the regulations of the University of London. These tables are indicated in italic fonts *'CD'* in the text.

1 Background

1.1 Definition of Subfertility

Fertility is defined by the duration of exposure to the chance of conceiving. Epidemiological data indicate that the cumulative conception rate in women aged 25 years or younger is 60% at six months, 85% at one year and 91% at two years[1,2,3].

By convention, couples at the lower end of the normal distribution of fecundity who do not conceive within one year will be included in the subfertile group, although as a random event conception may be delayed by chance alone[4,5,6]. Therefore, it is important to recognize that any epidemiological demarcation between fertility and subfertility is arbitrary. However, as the duration of exposure to the chance of conception increases, it becomes less likely that couples will remain subfertile due only to chance factors with no underlying pathology, since self-selection lowers the natural fertility of the residual non-pregnant population[7].

1.1.1 Prognostic Factors in Assisted Conception

Female age is the most important determinant of live birth rates following IVF treatment. After adjustment for female age there is a significant fall in live birth rates with increasing duration of subfertility and repeated failed IVF cycles. Previous pregnancy (especially IVF pregnancy) and childbirth enhance treatment success. The cause of subfertility has minimal effect on the outcome[8].

However, assessment of specific prognostic factors that might affect the treatment outcome should not be performed in isolation and the overall success rate of the treating clinics should also be evaluated. Mostly these variations are attributed to the expertise of the individual clinics. It is reported that even within a single clinic the success rate may vary from one physician to the next[9,10]. It is recognized that, with a higher number of treatment cycles, larger clinics generally will perform better than smaller clinics[11]. This is possibly due to economies of scale allowing

opportunities for better quality control, and economies of scope where clinics can provide a wider selection of treatment options to tailor the fertility management of individual couples. However, the importance of size to explain the inter-clinic variance has been challenged[12]. The other widely accepted argument to explain such persistent differences between clinics is the dissimilarity in the proportion of good and poor prognostic couples in their treatment populations[13].

1.1.1.1 Female Age

Several authors have constructed statistical models to predict the probability of pregnancy after embryo transfer (ET) using multivariate analysis of prognostic factors. The consistent conclusion is that the age of the female partner is the single most important factor determining spontaneous fertility and the outcome of fertility treatment[8,14,15,16,17,18].

Commenges-Ducos et. al.[19] studied 923 IVF cycles and found that women aged >38 years had less success in IVF because they have a lower probability of producing oocytes and of implantation of embryos. In spite of increased dosage of human menopausal gonadotrophin (hMG) stimulation older women yielded fewer oocytes and had a higher cancellation rate[20]. The striking finding is that female age does not influence the probability of fertilization, which is in agreement with Padilla and Garcia[21].

Using logistic regression on a national database established by the Human Fertilisation and Embryology Authority in the United Kingdom, Templeton et. al.[8] demonstrated that live birth rates were highest in the group aged 25-30 years with a sharp decline in older women. Templeton et. al. model the effect of age with a nonlinear continuous curve showing only a slight decrease in the probability of success until aged 35 years, followed by a sharp decrease after aged 35 years[8]. A steeper decline after the age of 37 years was reported by Van Kooij et. al.[22]. Stolwijk et. al.[23] found a simple linear effect. This agrees with the results of other national database reports from the United States and France[11,24,25,26,27].

It is generally accepted that decline in fecundity with increasing age is due to the decreased fertility potential of oocytes[28,29,30] rather than a specific uterine factor. The aging uterus is shown to respond adequately to the circulating oestrogen and progesterone[31]. Navot et. al.[32] found no difference in implantation rates between younger and older donor oocyte recipients undergoing identical hormone replacement protocols.

It is proposed that decline in the overall quality of oocytes with advancing age occurs due to the development of chromosomal defects. It is now well established that reduction in the number of follicles occurs simultaneously with the development of meiotic spindle abnormalities in oocytes from older women[33], as with the aneuploidy of embryos[34] and accelerated granulosa cell apoptosis[35].

This chromosomal defect can be congenital. As female age advances the follicular pool in the ovaries decreases as a result of ovulation and physiological atresia[30,36]. During this process oocytes with congenitally defective chromosomes are not recruited until the healthy oocytes are utilised. Hence, the defective oocytes start dominating the pool in the later reproductive years[37,38,39]. Alternatively, as a part of the general physiological aging of the body, chromosomal defects can be acquired secondary to prolonged exposure to the risks of accidental damage[40]. Whatever the mechanism, the available oocytes are poorer in quality with advancing age[41].

1.1.1.2 Duration of Subfertility

Increased duration of fertility delay is associated with a reduced possibility of spontaneous conception in untreated subfertile couples. Similarly, duration of subfertility is prognostic for treatment-related conception rates. A logistic regression model constructed from the Human Fertilisation and Embryology Authority (HFEA) database of 36,961 registered cycles shows a significant reduction in the live birth rate with increasing duration of subfertility even after adjustment for female age. While three years of subfertility is associated with a live

birth rate of 15.3% after IVF, 10 years of subfertility drops the live birth rate per treatment cycle to 12.4%[8].

1.1.1.3 Previous Pregnancy History

Analyses of the HFEA database highlight that any pregnancy will increase the chances of live birth after IVF treatment even after adjusting for female age. This effect is stronger with a history of previous live births than for a history of pregnancies not ending in live birth, especially when the previous pregnancy resulted from IVF treatment[8].

An analysis by the French National Register on IVF (FIVNAT) of all oocyte collections between 1989 and 1995 also reveals that women with no previous IVF pregnancy had lower success in response to ovarian stimulation, fertilization and pregnancy rates, when compared with women with a previous failed IVF pregnancy (spontaneous abortion or ectopic pregnancy) or ongoing IVF pregnancy[42].

Other investigators report similar findings[20,43]. A database review of 5073 first and 2396 subsequent IVF cycles shows that even a prior biochemical pregnancy is a good prognostic indicator[20]. In this series, a similar clinical pregnancy rate of 36.4% in second treatment cycles is reported following both a biochemical and clinical pregnancy in the first cycle. Human chorionic gonadotrophin (hCG) rises, although transient, are claimed to indicate the presence of an embryo sufficiently well developed for implantation[44].

1.1.1.4 Previous Treatment Cycles

In a study using the HFEA IVF database, it is demonstrated that the live birth rate per treatment cycle is highest in the first cycle and decreases significantly with increasing numbers of previous treatment cycles, after adjusting for female age[8]. The same finding is reported by the French Registry of IVF, which has built a cohort of 35,714 couples since 1986. The clinical pregnancy rate per oocyte collection decreased from 20.2% in the first attempt to 17.4% in the second, and to

<13% after the sixth. This trend persisted whatever the female age or subfertility diagnosis[27]. However, there appears to be no consensus in the literature.

Croucher et. al.[20] shows that the 26% chance of pregnancy after a single IVF cycle remains constant for the first three attempts and only subsequently does this begin to decline. Cumulative pregnancy rates continue to rise, albeit at slower rates; 54% after three cycles and 72% after six. Some studies indicate a constant rise in the cumulative pregnancy rate up to six cycles, where it reaches a plateau of 56%[45,46,47], and even up to eight cycles[48].

This indicates a self-selection process occurring throughout repetitive treatment attempts, during which the proportion of couples with a good prognosis is declining. This leads to a progressive fall in overall success rates in the residual non-pregnant population undergoing IVF treatment. The speed of this decline is a function of the characteristics of the initial cohort, which is different in each study.

1.1.1.5 Cause of Subfertility

In contrast to the prediction models of untreated couples where spontaneous live birth rates are strongly affected by seminal defects, tubal disease, and endometriosis, the HFEA database demonstrates no association between different causes of subfertility and the outcome of IVF treatment in terms of live birth rate per treatment cycle[8]. A similar outcome is reported by Croucher et. al.[20], who report that even when multiple factors are involved, the cause of subfertility does not affect IVF outcome.

However, the national HFEA register database analysis reveals a different picture when the live birth rates are calculated per ET rather than per cycle initiated[8]. Unexplained subfertility (19.7%) achieved a higher success rate than tubal factor subfertility (16.5%) and endometriosis (17.9%). This difference is explained by either lower fertilization but higher implantation rates in unexplained subfertility, or lower implantation rates in tubal subfertility. The presence of hydrosalpinges may

contribute to the negative influence of tubal subfertility; nevertheless, severe tubal damage, regardless of hydrosalpinges, is associated with poor IVF outcome[49,50].

Some authors find a reduction in pregnancy rates among women with endometriosis due to functional impairment at different stages of fertility. Altered folliculogenesis[51], ovulatory dysfunction[52], hyperprolactinemia[53], luteal phase defect[54], accelerated ovum transport[55], sperm phagocytosis[56], impaired fertilization[57], poor embryo quality and embryo toxicity[58], and defective implantation[59,60] are suggested as possible mechanisms of reduced fertility in endometriosis.

However, these observations are not universal. The presence of endometriomas in women undergoing ovum donation does not appear to affect implantation[61]. Results from the large national databases in the UK and US[8,11,24,25,26] do not support the contention of lower pregnancy rates with endometriosis. Despite their power to overcome the problems of study population size, found in smaller but better controlled studies, the inherent problem of national databases is their inability to collect detailed information regarding the stage of endometriosis. Therefore, the pooled summative review may miss an association at a subgroup level.

Although male factor subfertility is recognized to compromise the treatment outcome in earlier reports from IVF clinics[14,15] or national databases[27], this apparent disadvantage is no longer valid due to the introduction of the intracytoplasmic sperm injection (ICSI) procedure into routine fertility management[8,11,26].

1.1.1.6 Ovarian Reserve

In terms of fertilisation and subsequent implantation, oocyte quality determines fecundity and correlates well with the total number of oocytes in the ovary. As direct oocyte assessment at clinical level is not feasible outside the context of IVF,

follicular characteristics have been used as surrogate parameters in ovarian reserve tests. These tests have been designed to reflect the quantity of the remaining follicular pool in the ovary and so signify the quality of the oocytes.

During the first five days of an ovulatory cycle, basal serum follicle stimulating hormone (FSH) levels reflect the number of antral follicles ready for recruitment[62]. Rising levels of early follicular phase FSH reveal a reduction in the size of this follicular cohort and a subsequent reduction in inhibin-mediated negative feedback in the pituitary[30,31,36].

As is evident in clinical practice, while FSH levels increases with decreasing ovarian reserve the follicular response to gonadotrophin stimulation also reduces, with fewer follicles developing, fewer oocytes produced, and fewer embryos created[63,64,65]. This observation is valid even when the rise in FSH levels occurs within the normal range and is found to be chronologically independent and in advance of female age[66,67,68]. Supporting this finding, it is reported that basal FSH levels provide a much better predictive power for pregnancy rates than female age[69]. Faddy et. al.[41] conclude that the ovary measures biological time according to the number of oocytes remaining, rather than chronologically.

In an IVF setting, El-Toukhy et. al.[70] demonstrate that treatment outcome does not differ in poor responders aged ≤30 years when compared with their older peers for fertilization, implantation, pregnancy and live-birth rates. It is proposed that these women, regardless of age, share an inherent oocyte problem referred to as 'ovarian ageing'[71,72]. This contention is in contrast to studies suggesting that within poor responder groups, young women have a significantly better outcome than older women[73,74].

Although there is such a decline in the numbers of oocytes recruited by exogenous gonadotrophin stimulation in women with high FSH levels[66,75], there appears to be no reduction in the proportion of these oocytes reaching maturity[76], or in their

fertilization, cleavage[71,77], or ability to produce high quality embryos[76,78]. However, the implanting ability of individual embryos declines with female age and thus pregnancy rates[75,79]. It is argued that chromosomal abnormalities of the oocyte need not affect fertilization and early embryo cleavage but can be linked to failure of implantation[80,81,82].

Inter-cycle variation of gonadotrophin concentrations is well documented but these fluctuations are considerably less in women with low FSH concentrations[83,84]. It appears that the inter-cycle variability in basal FSH levels generally does not affect the woman's prognostic category[85] and a single raised FSH value, even if subsequent levels are normal, is associated with decreased ovarian responsiveness, fertilization and implantation rates[85,86].

Post-test probabilities of elevated basal FSH concentrations enable us to identify women who can be advised not to undergo IVF treatment, but only where a significantly high cut-off level is chosen[68,86]. This is because, despite having a high specificity, basal FSH levels have limited sensitivity[87]. A substantial group of women will have normal FSH and yet respond poorly to stimulation and have poor pregnancy rates. This is explained by proposing that the FSH level is a late marker of decreased fertility potential[88,89].

Immunoassays of luteinising hormone (LH) and FSH are intrinsically imprecise and differences in the antibodies used to measure gonadotrophin levels contribute to this imprecision. Most of the commercially available assays use polyclonal antibodies that bind differently to separate haptens on the glycoprotein hormone. Consequently, as the distribution of different isoforms changes throughout the menstrual cycle, the ability of any single assay to recognize the gonadotrophins present may differ substantially. The assay that reports a relatively higher value with one set of isoforms could present a lower value when measuring another. In addition, different assays may be calibrated against different reference preparations, adding further variability to the reported results[87].

1.1.1.7 Number of Embryos Transferred

The number of embryos transferred affects the chances of a live birth, but the implantation potential of the individual embryo, which can be proximated by the total number of embryos available for transfer, seems to be more important[90]. If a high number of embryos is available for transfer this is shown to enhance the likelihood of a live birth, whereas when only one or two embryos are available for transfer there is a reduced chance of success. However, transferring high numbers of embryos with high implantation potential carries an increased probability of multiple pregnancy and this is one of the major adverse effects of IVF treatment.

The current debate is on how to minimize the probability of multiple pregnancy without jeopardizing the likelihood of a healthy singleton birth. An analysis of a large national database provides the answer to this[90]. Guidelines were formulated based on recognition of the fact that the number of oocytes fertilized and the number of embryos available for transfer is an important factor in determining the outcome. Where more than four embryos were available for transfer, the live birth rate was not affected by the transfer of two or three embryos, although there was a significant reduction in the multiple pregnancy rate when two embryos were transferred. This is shown to be valid for all female ages up to 40 years.

This recommendation has been criticized on the basis that no allowance is made for the possibility of varying embryo quality[91]. The ability to select suitable embryos remains an important determinant in the outcome of IVF treatment[90,92,93]. However, given the limitations of current embryo grading methods, availability of more than four embryos suitable for transfer may still inherently designate a higher level of ovarian reserve and a potential for better embryo quality.

Schieve et. al.[94] conclude that the probability of multiple pregnancy from IVF treatment varies not only by the number of embryos transferred but also by female age. Among women aged <35 years, maximum live-birth rates were achieved with as few as two embryos transferred. However, unlike the UK study[90], this

retrospective cohort analysis indicates a statistically insignificant increase in live-birth rates among women aged >35 years if more than two embryos are transferred, but at the expense of a highly significant increase in multiple birth rates from 12% to 29%.

Female age is a well-documented prognostic factor in embryo quality[95] and treatment outcome[8]. Although live-birth rates drop with advancing age, the effect of age on multiple pregnancy rates is not strong enough to relax the recommendations for the number of embryos transferred. In this context, the research based data have challenged the perceived sense of security regarding the low multiple pregnancy probability of women aged in their 40s[96,97,98].

From the earlier studies by Staessen et. al.[92,93,99] on the effect of number and quality of embryos transferred on multiple pregnancy, to the retrospective case control study by Licciardi et. al.[100], and the data analyses of UK IVF clinics by Ozturk and Templeton[101], the same trend has consistently emerged. Reducing the number of transferred embryos from three to two largely eliminates the occurrence of triplet pregnancies without altering overall pregnancy rates. The British Fertility Society and the Royal College of Obstetricians and Gynaecologists recommend that UK clinics observe a limit of two embryos per transfer[102]. Recently the HFEA have also recommended that only two embryos should be transferred. However, as these studies highlight, such a policy does not address the high prevalence of twin pregnancies, which are more frequent than higher-order multiples and contribute substantially to perinatal morbidity. If the ultimate goal of IVF is the birth of a single healthy child, the way ahead is proposed to lie with elective single-ET with the promise of subsequent transfer of frozen-thawed embryos[103] .

1.1.1.8 Embryo Quality

Embryo quality is a term used in clinical practice to describe an attempt to quantify the development potential of embryos *in vitro*. Estimation of embryo quality is used so that the best embryos can be selected for transfer after culture *in vitro*. The

pregnancy rate, implantation rate, and incidence of multiple pregnancies increase significantly with the number of good quality embryos transferred[92]. Embryo morphology is important in predicting the likelihood of embryonic progression. It is apparent that severe fragmentation is a poor prognostic sign. Blastomere size that is inappropriate for a particular stage of cleavage may indicate partial or incipient cleavage arrest. The multinucleation and timing of the earliest stages of cleavage are among the many methods promising more sensitive assessment, but to date there is no reliable method of predicting developmental competence or implantation potential with any degree of accuracy[104,105,106].

1.1.1.8.1 Embryo Selection Methods

The functional potency of human oocytes and embryos is a continuum that can be traced from prior to ovulation. As direct assessment of the developing oocyte in vivo is not possible, biochemical analysis of follicular fluid and sonographic evaluation of ovarian stromal and perifollicular blood flow have been utilised to inspect oocyte quality for fertilisation and implantation potential[107]. The grading of cumulus-coronal morphology is proposed as a useful predictor of success[108]. The presence of the first polar body in the perivitelline space was looked at to ensure the timely process of meiotic divisions and so the nuclear maturity of the oocyte[109,110]. The cytoplasmic polarity in human oocytes may also be linked to the potency of future development[111].

Early assessment of embryo morphology at the pronuclear stage provides the opportunity to evaluate the transient markers of embryo development that possess prognostic significance[112,113]. However, to date the embryo morphology on day 2 or 3 after insemination is the most frequently used parameter to assess the growth potential of individual embryos. The number of blastomeres is used to identify the stage of development of embryos, and relative size and shape of blastomeres and evidence of fragmentation are used to grade the embryos[92,96,114,115,116,117,118,119]. In addition to cross-sectional morphological evaluation, kinetic parameters of embryonic growth are utilised in relation to the timing of the first cell division[120] and

cleavage rate 48 hours after oocyte collection[92,121]. Estimation of embryo quality after three days of *in vitro* culture[122] or at the morula or blastocyst stage[123] takes advantage of the natural self-selection process for survival.

1.1.1.8.2 Cleavage Rate

The rate of embryo development is proposed as prognostic for future viability and more likely to be influenced by factors intrinsic to the oocyte and embryo rather than culturing protocols, female age, stimulation protocol and semen characteristics[114,124]. Early cleavage to the two-cell stage within 25 hours post-insemination is associated with significantly more clinical pregnancies[120].

Number of blastomeres at the time of transfer also acts as a proxy measure of the developmental potential of the embryo, linking closely to the embryonic genome activation that occurs between the four-cell and eight-cell stages of pre-implantation development[125].

Two studies that employed transfer of homogeneous quality embryos[116,126] report that no delivery was achieved using one-cell embryos or embryos obtained after delayed fertilization. Higher pregnancy rates were obtained with embryos displaying no irregular cells and embryos displaying no fragmentation. The four-cell embryos implanted twice as often as embryos with less or more cells[116,126]. Hsu et. al.[122] establish that despite embryo cleavage and morphology being strongly correlated, embryo cleavage is a superior indicator of implantation when compared with embryo morphology.

Van Royen et. al.[105] characterise a top quality embryo as four or five blastomeres on day 2 following fertilisation and at least seven on day 3, <20% fragmentation and no multinucleated blastomeres at any stage. In this series, 82% of all documented implantations were attributable to such top quality embryos. However, embryos with two blastomeres and initial slow development on day 2 following fertilisation have a high ongoing implantation rate if they later show an accelerated

growth rate with at least seven blastomeres on day 3, ≤10% fragmentation and no multinucleated blastomeres. Embryos with the same cleavage rate but with 10-20% fragmentation do much worse[106].

1.1.1.8.3 Cellular Fragmentation

Fragmentation is one of the most common morphological features used in the assessment of embryo quality. It is the extrusion of the plasma membrane and cytoplasm of an embryo into the extracellular space. Complete or partial fragmentation of blastomeres is a common occurrence during the early cleavage stages of human embryonic development, both in vivo and *in vitro*[127]. It is speculated that the process may be due to lethal defects in arrested embryos with a deleterious outcome[92,104,116]. However, fragmentation may also be associated with self-protecting events[128] and the embryo maintains its viability by eliminating affected blastomeres through apoptosis, which leads to cellular fragmentation[129,130].

Staessen et. al.[99], Giorgetti et. al.[116], and Ziebe et. al.[126] report low implantation rates after transfer of embryos with 10-50% fragmentation on day 2. However, these reports do not consider the size and distribution of the fragments. Alikani et. al.[130] have addressed this concern and their findings indicate that not all fragmentation, even at high levels, is detrimental to the embryo. Small, scattered fragments did not appear to negatively affect cell numbers and posed no serious threat to further development, as opposed to larger fragments. Ebner et. al.[131] detected significantly more congenital malformations in embryos with >25% fragmentation.

1.1.1.8.4 Multinucleation

Multinucleation of human pre-implantation blastomeres is caused by changes in cleavage patterns that can arise from karyokinesis (nuclear division) in the absence of cytokinesis (cellular division). Multinucleation can also arise by partial fragmentation of nuclei, or by defective migration at mitotic anaphase[132,133,134]. A

possible role of exuberant response to ovulation induction[135], or suboptimal culture conditions with a detrimental effect on cytoskeleton function is proposed in the development of multinucleation[132,134,136].

Van Royen shows that of 213 embryos with documented ongoing implantation, there was only one embryo with multinucleated blastomeres[105]. Tesarik et. al.[137] found that multinucleated embryos can cleave beyond the eight-cell stage but these cells have defective transcription of genes that eventually leads to developmental arrest. If this happens after implantation it results in early pregnancy loss. Several authors advise against transfer of multinucleated embryos because of their poor developmental potential and high rates of chromosomal abnormalities[132,138,139].

1.2 Aspirin

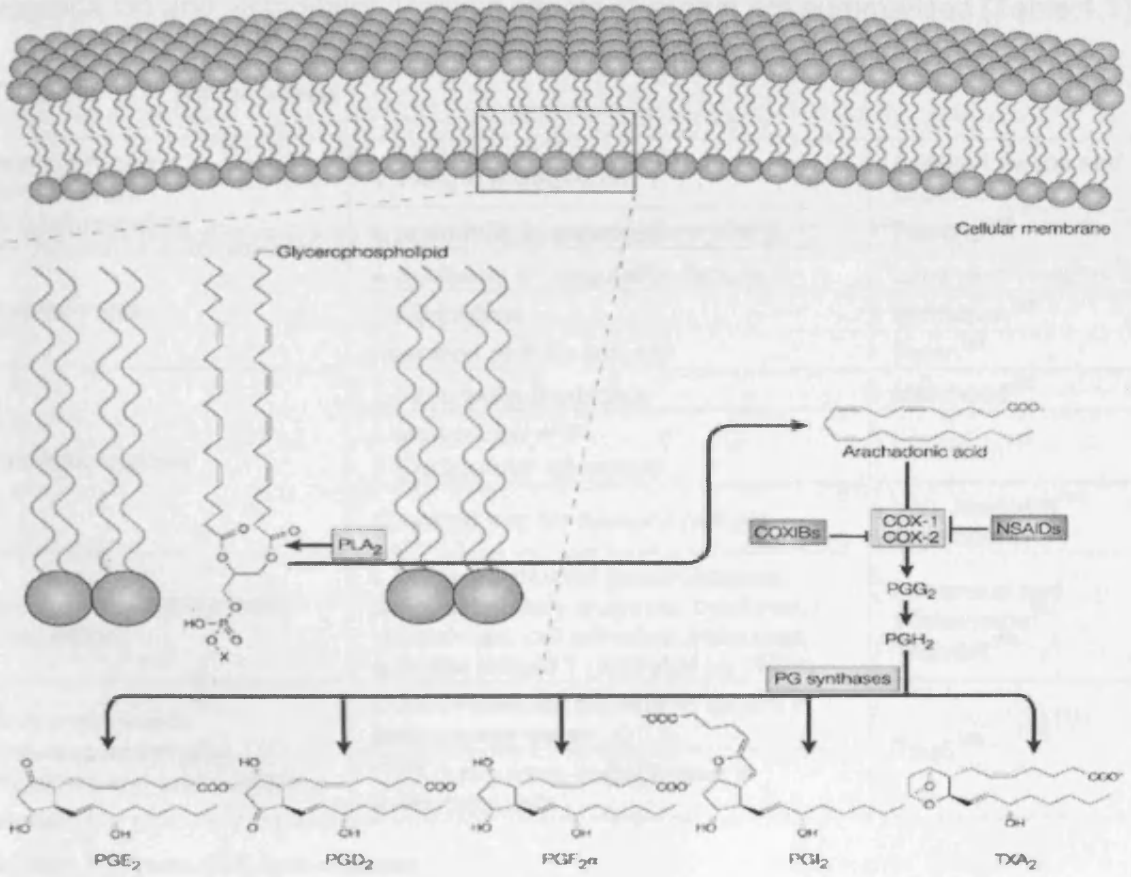
Aspirin is a product of the late 19th century but medical historians have traced its origins to 3,500 years ago. The Ebers papyrus, a collection of 877 medicinal recipes from the second millennium BC, recommends an infusion of dried myrtle leaves for rheumatic and back pain. One thousand years later, Hippocrates of Kos prescribed a juice extracted from the bark of the willow tree to relieve the pain of childbirth and to reduce fever. All of these medicinal remedies contain salicylates and the name is derived from salix, the Latin word for willow.

In Germany in 1860, salicyclic acid was chemically synthesized as an external antiseptic and commonly used for the treatment of rheumatism. Subsequently, on 10th August 1897, the active ingredient, acetylsalicylic acid, was synthesized for the first time in a chemically pure and stable form by Dr Felix Hoffmann, a chemist working for Bayer, who was urged by his father to make a more palatable form of salicylate to treat his rheumatism.

1.2.1 Mode of Action

Aspirin covalently and irreversibly modifies both cyclo-oxygenase -1 and -2 (COX-1 and COX-2) by acetylating Serine-530 in the active site[140,141]. Acetylation of COX-1 creates a steric block that prevents the binding of arachidonic acid at the COX active site (Figure 1.1). Acetylation of COX-2 retains the COX activity although the reaction produces a novel product 15-R-HETE, which serves as substrate for the formation of 15-epi-lipoxin A_4, a compound that can exhibit anti-inflammatory properties primarily by down-regulating granulocyte activity[142,143].

Figure 1.1. Prostaglandin synthesis



Aspirin can be regarded as a non-selective COX inhibitor, although it possesses higher potency for COX-1[144]. Aspirin does not inhibit lipoxygenase. On the contrary, inhibition of COX may lead to increased formation of leukotrienes, most

likely because of an increase in the amount of free arachidonic acid available to the lipoxygenase enzyme.

Acetylation of COX by oral aspirin is dose-dependent, cumulative and selective with repeated administration. However, biochemical selectivity is at best relative but not absolute. Various regimens of low dose aspirin (LDA) were tried to achieve maximum Thromboxane A_2 (TxA_2) suppression while maintaining prostacyclin (PgI_2) levels in both obstetric and non-obstetric populations. Thromboxane A_2 has two potent actions: it stimulates both platelet aggregation and release reaction, and also contracts blood vessels. Prostacyclin is a potent inhibitor of platelet aggregation and vasodilator. Various effects of aspirin are summarised (Table 1.1).

Table 1.1. Aspirin effects

Anti-aggregation Anti-thrombin Anti-inflammatory	↓ TXA_2 in platelet-COX 1	Catella-Lawson and Crofford[145]
	↓ or no PGI_2 in endothelium-COX 2	Patrono[146]
Anti-thrombin	↓ Synthesis of coagulation factors	Loew and Vinazzer[147]
	↑ Fibrinolysis	Bjornsson[148]
	Alteration of fibrin porosity	Fatah[149]
Anti-inflammatory	↑ Nitric oxide production	Mahmood[150]
	↓ Intracellular ATP ↑ Extracellular adenosine	Cronstein[151]
	Chemical trap for hydroxyl radicals	Van Jaarsveld[152] Colantoni[153]
Immune and inflammatory modulation	↓ Mitogen activated protein-kinases, pro-inflammatory enzymes, cytokines, chemokines, cell adhesion molecules, activator protein 1 (activated by TNFα)	Abramson and Weissmann[154] Tegeder[155]
Anti-angiogenetic Prevents endothelial migration and tube formation	COX-2 modulate angiogenic factors in colon cancer cells	Tsujii[156]
	COX-1 regulates angiogenesis in endothelial cells	

↓: Lower, ↑: Increase, COX: Cyclo-oxygenase

1.2.2 Aspirin Regimens

Two strategies have been proposed to enhance the biochemical selectivity of aspirin for TXA_2: a reduced dose and an alternate-day regimen. At low doses most of the aspirin is metabolized on the first pass through the liver. Thus, little or no

aspirin reaches the systemic circulation to impair vascular endothelial PGI_2 production while repeated daily doses assure that virtually all platelets are exposed to aspirin at perihepatic circulation. Dosing with aspirin on alternate days is based on the premise that, unlike platelets, endothelial cells retain the capacity to generate new enzyme despite the irreversible inhibition of prostaglandin G/H synthase. Although the enzyme remains inhibited for the lifetime of exposed platelets, enzyme turnover permits functional recovery in the vascular endothelium *in vitro*. In addition, platelet COX may be more sensitive to aspirin inhibition than the vascular enzyme.

With a single daily administration the aspirin dose for near-complete suppression of serum thromboxane formation with best differential sparing of prostacyclin has been titrated downward to approximately 0.5-2 mg/kg[157]. Urinary excretion of a stable hydrolysis product of prostacyclin still fell by 20% during the first four days of the treatment (0.5 mg/kg) but this failed to attain statistical significance[158]. The fall in PGI-metabolite excretion attained significance at doses in excess of aspirin 160 mg/day[159].

The antiplatelet effects of the daily dose of aspirin depend on the time pattern of administration[160]. Intermittent pulsed rather than split administration of the same cumulative dose of aspirin favour both better inhibition of platelet function and preservation of prostacyclin formation. Serum thromboxane B_2 concentrations were nearly identical during treatment with aspirin 325 mg every third day or aspirin 81 mg every day[161].

Tulppala et. al.[162] evaluated the effect of low-dose aspirin on PGI_2 and TXA_2 production and on pregnancy outcome in recurrent miscarriages with and without anticardiolipin antibodies (ACA). Treatment with aspirin inhibited platelet TXA_2 production similarly in women with and without ACA and with ongoing pregnancies or miscarrying pregnancies. The administration of aspirin 50 mg during early pregnancy significantly reduced TXA_2 production but left PGI_2 output unchanged.

However, it did not improve pregnancy outcome in women with or without detectable ACA.

1.2.3 Safety Issues During Pregnancy

Aspirin is the most frequently ingested drug in pregnancy[163]. Studies investigating the effect of aspirin on hypertension in pregnancy and intrauterine growth retardation did not observe aspirin-induced fetal or neonatal toxicity after the chronic use of low-dose aspirin at 40 to 150 mg/day.

The possible relationship between aspirin in early pregnancy and congenital defects remains controversial. A Food and Drug Administration (FDA) surveillance study involving 1,709 newborns exposed to aspirin during the first trimester does not support an association between the drug and congenital defects[163]. The Collaborative Perinatal Project of 14,864 mother-child pairs, who used aspirin during the first trimester, found no evidence of a teratogenic effect in pregnancy[164]. A meta-analysis has shown that the probability of congenital malformations in the offspring of women exposed to aspirin in early pregnancy is not significantly higher than that in control subjects (odds ratio, 1.33; 95% Confidence Interval (CI), 0.94-1.89)[165]. However, a significantly increased probability of gastroschisis (odds ratio, 2.37; 95% CI, 1.44-3.88) has been reported in women who have taken a nonsteroidal anti-inflammatory drug (NSAID) including aspirin and paracetamol during the first trimester of pregnancy[165,166].

A Danish population based case-control study of 1462 pregnant women prescribed NSAIDs at doses equivalent to 400 mg or 600 mg of ibuprofen shows that use of NSAIDs during pregnancy does not increase the probability of adverse birth outcome in terms of congenital abnormality, low birth weight, or preterm birth, but is associated with increased probability of miscarriage (odds ratios range from 1.3 for NSAID use 10-12 weeks before miscarriage to 7.0 for NSAID use one week before miscarriage)[167]. The study was criticised on the basis of incomplete evaluation of both the NSAID use during pregnancy and the miscarriages, and lack

of information on important confounders such as the reason for NSAID use. Li et. al.[168] also evaluate the prenatal use of NSAIDs in a population based prospective cohort study, revealing an increased probability of miscarriage associated with prenatal use of NSAIDs or aspirin. The associated probability was higher when NSAIDs were used around the time of conception and when used for longer than a week, indicating a dose-response relationship, though no precise dose information was available. In contrast to Nielsen et. al.[167], Li et. al.[168] identify the indications for use of the drugs, control the data for confounding factors and evaluate the effect of timing and duration of drug use. They conclude that the association between NSAID use and miscarriage is unlikely to be due to the underlying indications for use of NSAIDs or aspirin. Potential for biased recall and a confidence interval that includes 1.0 suggest that their results are on the borderline of statistical.

1.2.4 Safety Issues Relating to Bleeding Complications

Although bleeding time is prolonged during low-dose aspirin therapy, the clinical implications of this for the hemorrhagic complications of concomitant surgery do not seem to be universally accepted. Bleeding time does not correlate well with the occurrence of post-operative bleeding[169]. However, published clinical studies present conflicting data. An increased frequency of bleeding is reported after abdominal surgery in patients treated with indomethacin[170] and after prostate surgery in patients who received preoperative aspirin or other NSAIDs[171]. No significant increase in blood loss was found in patients receiving diclofenac concurrent with hip replacement, transurethral resection of the prostate, or gynaecologic laparotomies[172,173,174]. In a review of haemorrhage and transfusion requirements in patients undergoing coronary artery bypass grafting, it was concluded that aspirin does not predispose to significant hemorrhagic complications[175].

The risk of gastrointestinal haemorrhage from long-term treatment with sub-analgesic doses of aspirin is well acknowledged. A systematic review of randomised trials shows that the probability is 1.68 times higher based on an

average of 28 months' therapy. There was no evidence of dose responsiveness over a wide range (50 to 1500 mg/day) for the occurrence of this complication[176]. A multi-centre study concluded that 451 women who received low-dose aspirin (60mg) from 13 to 27 weeks of pregnancy onwards did not have any increased maternal bleeding complications or adverse effects related to epidural anaesthesia despite having increased bleeding time[177].

1.3 Antiphospholipid Antibody Syndrome

Antiphospholipid antibody (APA) syndrome presents with the clinical features of venous or arterial thrombosis, recurrent pregnancy loss, or thrombocytopenia. Diagnosis requires one clinical feature and at least one laboratory finding of either positive IgG or IgM anticardiolipin antibodies (ACA) >20 GPL units, or positive lupus anticoagulant (LA) test.

Defective embryo implantation is the unifying feature of all obstetric complications associated with APA[178,179,180,181]. The pathogenesis was initially believed to be associated with spiral artery thrombosis[182,183,184] and widespread placental infarction. However, later studies suggest that this is not a universal finding[185] and the focus has been more on the adverse effects of APA on embryonic implantation and trophoblast invasion.

Several possible mechanisms have been promoted. APA can modify the prostaglandin metabolism leading to a drop in prostacyclin levels or increase in thromboxane synthesis[186,187]. This synthesis might be normalized by aspirin[162,188]. Binding of APA promotes aggregation of platelets leading to thrombus formation and thrombocytopenia. APA also activates endothelial cells to express adhesive molecules and secrete pro-inflammatory molecules leading to vascular occlusive disease. Microscopically, fibrinoid necrosis and thrombosis of spiral arteries is noted and associated with extensive infarction in the placenta of women with recurrent pregnancy losses[182,183,189].

APA can bind to the surface of phospholipids resulting in direct cellular injury on the trophoblast and inhibiting the syncytia formation[179,190], giving rise to severe placental dysfunction. It is proposed that APA may interfere with phospholipid dependent gonadotrophin-releasing hormone induced signal transduction, and are able to affect human chorionic gonadotrophin secretion[191,192]. In human miscarriages associated with APA, the level of interleukin 3 (IL-3) stays low, which normally promotes trophoblast expansion and invasion[193]. ACA inhibit prolactin secretion and prostaglandin synthesis by human decidual cells[180]. The favourable effects of aspirin are shown to directly promote decidualisation[194].

A range of treatments comprising aspirin, heparin, steroids and intravenous immunoglobulin (IVIG) has been advocated to improve the poor live birth rate of women with APA syndrome. Women with a history of recurrent miscarriage and APA-positivity had a 90% miscarriage rate when they received no drug treatment, whereas a control group of APA-negative women with recurrent miscarriage had a 34% miscarriage rate[195,196].

The treatment of choice for pregnant women with APA syndrome is low-dose aspirin with heparin. Two prospective randomized studies report that this treatment combination leads to a 71% live birth rate in future pregnancies and is superior to low dose aspirin alone (42%)[196,197]. There was no difference in the live birth rate in the two-treatment modality in pregnancies that went beyond 14 weeks' gestation[195].

The antithrombotic effect of aspirin and heparin is well known. This explains their mode of action in recurrent miscarriages if one relies only on the concept of spiral artery thrombosis as the main factor of miscarriage in women with APA syndrome. However, aspirin may also increase the levels of interleukin-3, which promotes trophoblast invasion and trophoblast expansion[198]. Heparin also inhibits the binding of APA[199]. These actions may protect invading trophoblast phospholipids from damage. *In vitro* studies have reported that heparin restores placental human

chorionic gonadotrophin secretion and trophoblast invasion, both of which are adversely affected by APA[178,179].

1.3.1 Subfertility

The hypothesis of APA mediated pregnancy failure has been proposed to encompass not only clinically recognized failure of post-implantation pregnancy, but also failure of fertilization and implantation, and hence subfertility[200]. In otherwise clinically asymptomatic women, Gleicher and el-Roeiy[201] define this as 'reproductive autoimmune failure syndrome'. A general polyclonal B cell activation, possibly secondary to pelvic tissue damage of endometriosis or pelvic inflammatory disease[202], is claimed to be responsible for the clinical picture.

Information associating immune dysfunction with subfertility is largely confined to observational studies linking the prevalence of APA to clinical situations such as endometriosis, unexplained subfertility, pelvic disease and IVF failure.

Initially, the association between APA and subfertility is reported by Gleicher et. al.[203], Gleicher and El-Roeiy[201], and Taylor et. al.[204]. Subsequently, several other studies report a high prevalence, in the range of 15% to 59%, of APA in subfertile women undergoing IVF treatment [202,205,206,207,208,209,210,211,212,213,214]. Fisch et. al.[215], conclude that increased APA concentration in women undergoing IVF treatment reflects the subfertile state and is not secondary to the IVF treatment. Chilcott et. al.[216] report that the prevalence of APA is independent of the cause of subfertility (tubal, anovulatory, male factor, unexplained). Other studies report the incidence of APA in different patient populations: normal obstetric patients 5.3% (1.3-9.8), women with recurrent pregnancy loss 20% (8-37), women with systemic lupus erythematosus 37% (17-86). The prevalence of β_2GPI (Anti-beta$_2$-glycoprotein I) antibody, co-factor for ACA binding, was 16% in the recurrent miscarriage group and 9% in the IVF implantation failure group, compared with 0% in a control group of fertile women.

Although there are theoretical reasons to believe that autoimmunity may play a role in subfertility in some women, the evidence for association of abnormal autoimmune function with subfertility remains circumstantial. Any conclusion inferred from the published studies is fraught with inconsistencies among the study populations, different sets of autoantibodies used to establish diagnosis, absence of standardized autoantibody assays, and various definitions of 'normal' with regard to the autoantibody assays[217].

Hence, among women undergoing IVF for treatment of subfertility, the relevance of the presence of APA has been questioned. Although these women have a higher prevalence of APA, none of the studies assessing IVF parameters have identified decreased rates of oocyte collection, fertilization and embryo development and quality.

In order to evaluate whether among women undergoing IVF the presence of APA affects the likelihood of IVF success, Hornstein et. al.[218] published a meta-analysis of data collected from seven different IVF studies. This includes data on women with (n = 703) and without (n = 1350) elevated APA who underwent IVF treatment and, if APA-positive, received no therapy for abnormal levels of APA. Three studies were prospective[207,209,210] and four retrospective[202,219,220,221].

The aggregate clinical pregnancy and live birth rates are respectively 57.0% and 49.2% in the APA-positive women and 46.0% and 42.9% in the APA-negative women. The odds ratio describing an association between APA and IVF outcome ranged from 0.26 to 1.65 for clinical pregnancy and 0.19 to 1.64 for live births. No study reveals a statistically significant effect, although studies evaluating more antibody types are more likely to find a non-significant reduction in the likelihood of IVF success[202,209,210,219,220]. As estimated by the clinical pregnancy and the live birth rates, the presence of APA was not associated with reduced IVF success.

Since the meta-analysis was published, a prospective observational study by Chilcott et. al.[216] evaluated the prevalence of APA and anti-β_2-GPI antibodies and concludes that although women referred for IVF have a high prevalence of APA, these antibodies do not affect the outcome of treatment.

However, it is proposed that the apparent lack of association between the APA seropositivity and the outcome of IVF treatment does not disprove the alternative hypothesis of abnormal autoimmune function affecting fertility. The antibodies could be epiphenomenon and developing secondary to other immunological disturbances[212]. Therefore, the assumption that APA alone can predict poor IVF outcome would be fraught because APA are only one marker amongst many of an activated immune system, and may or may not exist in the presence of an abnormal autoimmune state.

1.3.2 Thrombo-prophylaxis in Subfertile Women with APA

Sher et. al.[202] studied the effect on the implantation rate in women undergoing their first IVF treatment, of administering low-dose aspirin (81 mg/day) and heparin (10000 International Unit/day). A significantly higher pregnancy rate is reported in treated APA-positive women (49%), than in untreated APA-positive women (16%) and untreated APA-negative women (27%). In a report [222] on the selective use of aspirin and heparin in combination with intravenous IgG, in the IVF cycles where aspirin and heparin were administered the live birth rate was significantly higher (45%) than when no aspirin and heparin was administered (17%). Women positive for anti-phosphatidylserine and/or anti-phosphatidylethanolamine had significantly lower birth rates than women with any other APA, 17% versus (vs) 43%. The live birth rate in these women was significantly improved when intravenous IgG was added to heparin and aspirin.

Not all investigators found aspirin and heparin treatment effective in improving the IVF outcome in APA-positive women. Three studies, two non-randomized[210,223] and one randomized, double-blind and placebo-controlled[224], report that aspirin and

heparin treatment does not improve IVF outcome in infertile women who are APA-positive.

1.3.3 Thrombo-prophylaxis in the General Subfertile Population

High resistance to uterine blood flow is associated with poor pregnancy outcome in women undergoing IVF treatment[225,226,227,228,229,230].

It is postulated that aspirin therapy may improve embryo implantation because of its effect on endometrial and ovarian perfusion by inducing a shift in local production of TXA_2 toward PGI_2. This could result in preferential delivery of gonadotrophins, other growth factors and metabolic substrates to improve folliculogenesis and oocyte quality. Similarly, development of the endometrium and final receptivity would be enhanced[228,231]. It is also argued that aspirin may block the PG-stimulated inflammatory process and the release of interleukins[232,233], which may lower the implantation rate. An improved prostacyclin/thromboxane ratio and reduced prostaglandin production may also prevent expulsion of the blastocyst while preparing the endometrium for implantation[234,235,236].

Before ET, the urinary excretion of TXB_2 metabolite is reported to be lower in women who achieved pregnancy than in those who did not[237]. Similarly, Battaglia et. al.[229] observed lower endometrial cell culture thromboxane concentrations in pregnant women than in non-pregnant women and this correlated directly with the Doppler flow indices of spiral arteries, whereby the PI values of the uterine and spiral arteries were significantly lower in women who became pregnant. Likewise, Hauth et. al.[238] showed that women assigned to receive aspirin had markedly lower maternal thromboxane B_2 concentrations than the placebo group. It has been shown that the blood flow velocity in the uterine and ovarian arteries increased significantly after ovulation induction[239].

Rubinstein et. al.[231], Urman et. al.[240], Kohl-Thomas et. al.[241], Tassa et. al.[242], and Salman et. al.[243] published their findings on aspirin co-treatment during fresh IVF cycles (Table 1.2). As seen in the table, these studies showed conflicting results.

Similarly, impaired uterine perfusion may be a common factor in frozen-thawed embryo replacement (FTER) cycles. A high proportion of women (37%) were found to have impaired uterine perfusion and hormone therapy alone does not invariably lead to good uterine perfusion[228]. Also, the published literature on aspirin co-treatment during FTER cycles is far from consistent (Table 1.2). In agreement with the findings of Goswamy et. al.[244], this study[228] found no difference in age and causes of subfertility between women with poor and normal uterine perfusion. When a long regimen with low-dose aspirin starting on day 1 of the hormone replacement therapy (HRT) cycle (47%) was compared with a short regimen with aspirin starting on day 13 (17%), Wada et. al.[228] report a higher pregnancy rate following FTER in women with poor uterine perfusion assessed by Doppler ultrasound. Although the low-dose aspirin on both the long and short regimens led to improved uterine blood flow, on the long regimen more women with poor uterine perfusion achieved improved perfusion. The advantage of the long vs short aspirin regimen is possibly due to more time available during the former to correct any imbalance in the thromboxane/prostacyclin equilibrium. The dose of aspirin selected for the short regimen (150 vs 300 mg/day) did not significantly affect treatment outcome. Other practitioners employed even smaller aspirin doses (50 mg/day) and observed improved utero-placental perfusion, thus preventing pregnancy-induced hypertension[245].

The high pregnancy rate (47% per ET) and low miscarriage rate (11%) observed in women with poor perfusion following treatment with the long aspirin-regimen, suggests that low-dose aspirin may benefit implantation and this is argued to be secondary to improved uterine perfusion[228]. Higher pregnancy rates were correlated with improved uterine perfusion and no advantage was noted in using aspirin in women with normal uterine perfusion. However, some of the women with

poor uterine perfusion failed to achieve improved perfusion, even when treated with the long regimen aspirin. In these cases, the reasons for treatment failure are uncertain and may be related to differences in the underlying cause of impaired perfusion.

Check et. al.[246] note no positive effect of low-dose aspirin on pregnancy rates following FTER. In this study, 18 women were treated with aspirin 81 mg from day 2 of the treatment cycle. In the aspirin group the clinical pregnancy rate was 11.1%, compared with 33.3% in the control group, and implantation rates were 2.9% and 10.9%, respectively. The mid-cycle mean endometrial thickness and echo patterns were similar in both groups. In the aspirin group 58% had no homogeneous hyper-echogenic pattern in the luteal phase, compared with only 28.6% of the control group. The mean PI was higher for the controls (3.0±0.7 vs 2.5±0.5). However, no inferential statistics were performed due to low numbers.

It is argued that for oocyte donation recipients with a poorly developed endometrium, the reduced pregnancy rates are attributable to decreased uterine perfusion. Weckstein et. al.[247] found that low-dose aspirin improves implantation rates in oocyte donation recipients with a thin endometrium (24% vs 9%). In women whose endometrium remained thin (<8 mm) in spite of augmented oestrogen stimulation, low-dose aspirin increased the pregnancy rate at a statistically significant level (83% vs 25%).

Beginning one week before starting oestrogen treatment, 28 recipients of oocyte donation who failed to develop an endometrial thickness of at least 8 mm during a test cycle of oral micronized oestradiol (E_2 2 mg for four days, 4 mg for four days, and 6 mg for four days), were randomized prospectively to receive low-dose aspirin (81 mg/day). Fifteen cycles were randomized to aspirin treatment, and 13 to non-aspirin treatment. Similar to the results of Check et. al.[246], the mean endometrial thickness in the aspirin and non-aspirin groups was not significantly different during

the test cycle, and there was no increase in the endometrial thickness despite aspirin therapy.

Uterine blood flow was not measured by Weckstein et. al.[247], but Check et. al.[246] documents no change in Doppler indices following aspirin therapy. In contrast, Kuo et. al. demonstrate that aspirin may improve uterine flow in women with unexplained subfertility and repeated treatment failures[248]. Based on a uterine artery Doppler pulsatility index of ≥3.0, one-third of the 127 women studied were found to have impaired uterine perfusion during their menstrual cycles. Those with impaired uterine blood flow were given aspirin 100 mg/day starting on day 3 of the next ovulatory cycle. The PI was measured in the natural and aspirin-treated cycles in the same group of women. A significant improvement in uterine blood perfusion was detected on the day LH peaked and in the mid-luteal phase (peri-implantation stage) of aspirin-treated cycles.

Table 1.2: Published Studies on Aspirin co-treatment in IVF

	Study Design	Number of Treatment Cycles	Aspirin Dose (mg)	Doppler	ORS	IR, PR
Rubinstein[231]	Randomised, Aspirin vs Placebo	149 vs 149 IVF	100 MLP	↑	↑	↑
Waldenstrom[249]	Randomised, Aspirin vs No aspirin	703 vs 667 IVF	75 ET	N/A		↑
Urman[240]	Randomised, Aspirin vs No aspirin	139 vs 139 ICSI	80 OS	N/A	↔	↓
Salman[243]	Randomised, Aspirin vs No aspirin	169 IVF, ICSI	81	N/A	↔	↔
Lok[250]	Randomised, Aspirin vs Placebo	30 vs 30 Poor responder IVF	80 MLP	↔	↔	↔
Pakilla[251]	Randomised, Aspirin vs Placebo	186 vs 189 IVF, ICSI	81 OS	N/A	↔	↔

ORS: Ovarian response to stimulation, IR: Implantation rate, PR: Pregnancy rate, ART: Assisted reproductive technology, FTER: Frozen-thawed embryo replacement, MLP: Mid-luteal protocol, ET: Embryo transfer, OD: Oocyte donation, OS: Ovarian stimulation, N/A: Not available, ↓: Lower, ↑: Increase, ↔: No effect.

Table 1.2: Published Studies on Aspirin co-treatment in IVF (continuation)

	Study Design	Number of Treatment Cycles	Aspirin Dose (mg)	Doppler	ORS	IR, PR
Kohl[241]	Retrospective	133 vs 92 ART	81 MLP	N/A	↔	↔
Tassa[242]	Retrospective	72 vs 244 IVF	80 MLP	N/A	↔	↓
Wada[228]	Randomised Aspirin vs Aspirin	26 vs 11 HRT for FTER Poor Perfusion	150 vs 300 Day 13 150 Day 1 vs 13	↑		↑ in Poor perfusion ↔ in Normal perfusion
Weckstein[247]	Randomised, Aspirin vs No aspirin	15 vs 13 HRT for OD Thin endometrium		N/A		↑
Check[246]	Randomised, Aspirin vs No aspirin	18 vs 18 HRT for FTER		↔		↓

ORS: Ovarian response to stimulation, IR: Implantation rate, PR: Pregnancy rate, ART: Assisted reproductive technology, FTER: Frozen-thawed embryo replacement, MLP: Mid-luteal protocol, ET: Embryo transfer, OD: Oocyte donation, OS: Ovarian stimulation, N/A: Not available, ↓: Lower, ↑: Increase, ↔: No effect.

1.4 Perfusion and Subfertility

Goswamy and Steptoe[252] were the first to suggest that abnormal uterine artery blood flow might be associated with subfertility. They related specific abnormal waveforms with failure of implantation in IVF patients and successfully improved uterine circulation and implantation with the use of oestrogen[244]. Subsequently, other authors confirmed a relationship of uterine and ovarian blood flow to unexplained subfertility[253,254] and to successful implantation following IVF[115,255,256,257,258,259,260].

1.4.1 Uterine Perfusion

1.4.1.1 Relationship to Oestrogen and Progesterone

Blood flow to the uterus increases in response to oestrogen, and the variations in uterine impedance over the normal menstrual cycle generally reflect the variation in circulating oestradiol levels[244,261]. A significant correlation between plasma

oestradiol level and endometrial blood flow exists in the follicular phase[262]. In the luteal phase there is no correlation between blood flow and oestradiol levels[263]. A significant decrease in impedance to uterine perfusion as a result of increasing progesterone concentration has been demonstrated, especially in the late luteal phase[264].

Oestradiol administered to postmenopausal women causes decreased resistance in the systemic circulation[265] and dilatation of the uterine arteries[266]. In some studies[267,268] progesterone opposes the effect of oestradiol but in others does not[266].

Zaidi et. al.[269] studied circadian rhythm in uterine artery blood flow and found a nadir in PI and a peak in time averaged maximum velocity (TAMV) at 06.00 h, following sleep.

1.4.1.2 Uterine Perfusion and Subfertility

Kurjak et. al.[253] noted absent end-diastolic flow during both the proliferative and luteal phases in subfertile women, but no difference in mean uterine artery resistance index (RI) between subfertile and fertile women. Tinkanen et. al.[270] reported that subfertility patients had high PI in both uterine and ovarian arteries in the luteal phase more often than fertile controls. Steer et. al.[254] studied uterine impedance on day 21 of a natural cycle and found a significantly higher resistance in various groups of subfertile women when compared with fertile women. The highest day 21 PI values were found in women presenting with anovulation but at a single point in time PI did not discriminate between the different groups of subfertile women. In the study population, there was no correlation of PI with age, number of years of subfertility, serum oestradiol level, or serum progesterone level.

1.4.1.3 Uterine Perfusion and IVF Outcome

Utilizing transvaginal colour Doppler ultrasound and based on differences in the mean uterine artery PI or RI, it is possible to distinguish between conception and non-conception cycles before ET. However, conflicting conclusions have been reached. Some authors have reported significant correlations between pregnancy rates and uterine artery Doppler flow values [115,230,244,255,256,257,271,272,273], but others have failed to show such a relationship [225,258,264,274,275,276,277,278,279,280,,281,282,283].

These conflicting results are explained by the multiple factors affecting the uterine artery PI and RI and it has been shown that uterine artery impedance may be different in women with different causes of subfertility[254,284]. Doppler indices are also affected by different ovarian stimulation regimens, different days of hemodynamic evaluation, and the circulatory levels of serum E_2. Guanes et. al.[285] and Check et. al.[286] found no difference in the average uterine artery PI or RI by age. Kurjak and Kupesic[287] conclude that the ageing process affects the uterus less than the ovaries in early postmenopausal years. However, a subsequent study found that in comparison to natural cycles the percentage reduction in the uterine artery PI during IVF was correlated with age, indicating that the benefits of ovarian stimulation in reducing uterine vascular impedance are less apparent in the ageing uterus[227].

Available data indicate a wide range of overlap in uterine artery PI values between pregnant and non-pregnant cycles[288,289,290]. Only a remarkably high vascular impedance (PI ≥3.3 and/or absence of diastolic flow) may predict impaired implantation; such a high impedance is detected in only 9% to 26% of non-pregnant cycles[256,273,288].

One of the explanations is that most of the blood passing through the uterine arteries never reaches the endometrium. In support of this contention, a poor correlation between PI in the uterine and spiral arteries has been demonstrated[289]

and it is suggested that a more logical approach would be to directly evaluate the vascularization around the endometrium to assess endometrial receptivity[282,291].

1.4.2 Endometrial Perfusion and IVF Outcome

Endometrial blood flow studies have utilised either conventional colour Doppler sonography[292,293] or the newer techniques of two- or three-dimensional power Doppler sonography[282,291,294]. Power Doppler sonography has the advantages of less direction dependence, higher sensitivity and better contrast of vascular contour[295,296]. In addition to qualitative studies, computer-assisted quantitative assessment of power Doppler vascular signals has also been applied[297,298,299] (Table 1.3).

At the myometrial-endometrial junction, a subendometrial area is identified as a thin hypo-echoic layer on ultrasound examination[300,301,302], and described as the subendometrial or junctional zone of the myometrium with distinct histology. Blood supply within 3 to 10 mm of the echogenic endometrial borders was studied as subendometrial perfusion[292,303].

Although most investigators agree that a high degree of endometrial perfusion shown by colour or power Doppler examination indicates a more receptive endometrium, there is no consensus on how to assess changes in endometrial perfusion during IVF-ET cycles. Those combining the endometrial and subendometrial flow parameters show significant differences between pregnant and non-pregnant women[291,293,294]. In contrast, where attention is focused only on intraendometrial or subendometrial blood flow there is no significant difference [274,283,304].

An immunocytochemistry study reveals that the subendometrial myometrium (junctional zone) exhibits a cyclic pattern of oestrogen and progesterone receptor expression parallel to that of the endometrium[305]. Thus, the endometrium and subendometrial myometrium may form a functional unit with various cyclic

reproductive functions. In the pregnant group the junctional zone became significantly thicker at ET; in contrast, changes in the junctional zone were less pronounced in the non-pregnant group[306]. In spite of the higher uterine artery PI and RI values in spontaneous cycles, Basir et. al.[309] detect no significant difference in the number of colour signals in the endometrium between spontaneous and stimulated cycles. It is proposed that the relationship between uterine artery blood flow and endometrial perfusion may not be linear. They also report that the number of women showing endometrial colour signals was significantly lower in high responders than in moderate responders. It is proposed that despite low uterine PI and RI values, endometrial blood flow and receptivity in high responders is impaired.

It is claimed that low vascular resistance in the ovarian arteries of high responders might cause shunting of blood from the uterus through the utero–ovarian collaterals to the ovaries and diminish endometrial perfusion. Increase in hormone concentrations in the peripheral plasma leads to a decrease in peripheral vascular resistance and a decreased contractility of the uterine muscles. This results in relaxation and opening up of the small uterine vascular channels[252,310].

The concept of evaluating uterine receptivity by a cumulative uterine score including the endometrial blood flow was first introduced by Applebaum[292]. Even in the presence of other favourable parameters, with the absence of subendometrial blood flow, no conception was achieved. By using a similar approach, Salle et. al.[279] calculated a uterine score in the secretory phase of the menstrual cycle preceding IVF. None of the individual ultrasonographic or Doppler parameters tested was of sufficient accuracy to predict uterine receptivity, but the uterine score seemed to be a useful predictor of implantation. Baruffi et. al.[311] also evaluated an ultrasonographic uterine scoring system on the day of hCG administration, that correlated with female age.

Table 1.3. Doppler Ultrasonography of Endometrium and IVF Outcome

Study	Doppler Parameters	Day of	Pregnancy
Zaidi[293]	Sub-endometrial PSV, PI	hCG administration	No significant difference between pregnant and non-pregnant
Yuval[274]	Endometrial Colour and Power Doppler PI, RI, S/D; Absence of diastolic flow	Oocyte collection ET	
Contart[304]	Power Doppler Sonography of endometrium	hCG administration	
Schild[283]	Absence of spiral artery blood flow	Oocyte collection	
Zaidi[293] Chien[307] Salle[279] Battaglia[229]	Presence of sub-endometrial and intra-endometrial vascularization	hCG administration ET Oocyte collection	Higher Pregnancy
Battaglia[229]	Lower endometrial cell culture thromboxane and lower spiral artery PI	Oocyte collection	
Yang[291]	Larger Power Doppler area of endometrium ≥5 mm^2	Day before oocyte collection	
Schild[282]	3-D Power Doppler Sonography of sub-endometrial area	First day of ovarian stimulation	
Kupesic[294]	Lower RI in sub-endometrial vessels by Colour Doppler ultrasonography and Higher flow index by 3-D power Doppler	ET	
Jinno[308]	Endometrial tissue blood flow by hysterofiberscopical laser blood-flowmetry values ≥29 mL/min per 100 grams of tissue	Between days 4 and 6 of the luteal phase in spontaneous cycle	

PSV: Peak systolic velocity, PI: Pulsatility index, RI: Resistance index, S/D: Systolic/Diastolic, ET: Embryo transfer, Min: Minute

1.4.3 Ovarian Blood Flow

Ovarian blood flow can be studied as either the ovarian artery flows towards the substance of the ovary[287] or by analysing the artery within the ovary itself as intra-ovarian stromal flow[259]. For such small vessels, the angle of insonation cannot be determined and hence the reproducibility of Doppler indices in intra-ovarian vessels has been questioned[312].

1.4.3.1 Relationship to Oestrogen and Progesterone

In cycles stimulated with hMG, the PI of the ovarian vessels supplying the dominant follicle and corpus luteum is positively correlated with serum oestradiol

concentrations, but not with serum progesterone[313]. It is suggested that oestradiol increased stromal blood flow, but not corpus luteum blood flow. Striginc et. al.[314] show that administration of progesterone 100 mg intramuscular (IM) during the luteal phase significantly suppressed uterine artery PI, but did not affect intra-ovarian artery PI. However, Glock and Brumsted[315] note a strong negative correlation between serum progesterone and intra-ovarian vessel RI during the mid-luteal phase. Engmann et. al.[278] show a significant decline in ovarian stromal flow velocity after two to three weeks administration of gonadotrophin releasing hormone agonist (GnRHa), but were unable to show a similar concurrent reduction in the uterine blood flow.

1.4.3.2 Ovarian Blood Flow and IVF Outcome

Zaidi et. al. were the first to show a relationship between ovarian stromal blood flow velocity and ovarian follicular response[316]. The ovarian stromal PSV was measured in the early follicular phase, showing that poor responders have low ovarian blood flow even after adjustment for age, polycystic ovarian syndrome (PCOS), serum FSH and the number of hMG ampoules used. It is also reported that PSV, but not PI, is related to subsequent follicular response[259].

Engmann et. al.[278] propose that deficient intra-ovarian vascularity may serve as the initial marker of reduced ovarian reserve and precedes an increased FSH level and reduction of the ovarian volume. They speculate that the ovarian stromal blood flow velocity after pituitary suppression is a true representative of baseline ovarian blood flow, because the ovaries are in a quiescent state.

The main shortcoming of these studies on ovarian stromal blood flow is the use of PSV, which requires knowledge of the angle of insonation to the blood vessels analysed. Ovarian vessels inside the ovarian stroma are thin and tortuous and it is impossible to accurately determine the angle between the ultrasound beam and intra-ovarian vessels.

Assessment of follicular blood flow has been suggested as an alternative predictor of ovarian responsiveness and treatment outcome[317]. Barber et. al.[318] report a highly significant difference in the RI values between patients who became pregnant and those who did not; no patient who became pregnant had an RI greater than 0.5 on day 3 after ET.

Nargund et. al.[319] report a significant correlation between follicular blood flow and oocyte collection, as well as embryo quality. The absence of follicular blood flow correlated strongly with failure to collect an oocyte. PSV was significantly higher in follicles associated with a good-quality pre-implantation embryo, when compared with follicles yielding oocytes that did not fertilize or produced inferior embryos. There was no relationship to PI.

Peri-follicular circulation was subjectively scored using a grading system based on the percentage of follicular circumference that depicted an echo signal on the day of oocyte collection (Grade 1 <25%, Grade 2 <50%, Grade 3 <75% and Grade 4 >75%) [320,321].

Mean follicular diameter, number of oocytes collected, number of mature oocytes collected and fertilization rates were all significantly higher and triploidy rate significantly lower in the cohort of follicles with high-grade vascularity. There was no correlation between embryo morphology and grade of vascularity [321]. The pregnancy rate for cycles where the embryos transferred were derived from follicles with uniformly high-grade vascularity (3 or 4 only) was significantly higher than for cycles where the embryos were derived from mixed (1 to 4) or low- (1 or 2 only) grade follicles. There were no significant differences in uterine artery or intraovarian PI values between the pregnant and non-pregnant treatment cycles. There was a trend to a lower rate of early pregnancy loss in cycles where the embryos transferred were derived from follicles with higher vascularity, but differences were not significant[321].

Coulam et. al.[322] assess the role of follicular vascularity in predicting pregnancy after IVF. Using transvaginal ultrasonography, PSV was measured from the three largest follicles on both the right and left ovaries on the day of hCG administration. The quality of follicular flow was graded from 1 to 4 according to the amount of visible colour flow around the follicle. All pregnancies occurred in women with Grade 3 and 4 follicular vascularity and 91% of pregnancies occurred with follicular PSV ≥10 cm/s. This threshold value of PSV predicted pregnancy with high sensitivity (91%) and negative predictive value (97%), but low specificity (36%) and positive predictive value (13%), suggesting that reasons not associated with follicular flow are associated with failure to conceive in this high-risk population.

A link between follicle size and vascularity is suggested with the finding that perifollicular peak velocity values gradually increase with the increasing size of the follicles[323]. Highly significant elevation of the peak velocity was observed especially after hCG injection. Bhal et. al.[321] also witnessed low vascularity grades in smaller follicles as opposed to high-grade perfusion in larger follicles and this may reflect the increased maturity of high-grade vascularity follicles. Balakier and Stronell[323] postulate that low-grade follicle vascularity pre-hCG may affect the uptake of hCG and result in impaired maturation of the cumulus-oocyte complex. Also, a higher grade of perfusion may lead to the increased access of FSH to those follicles, promoting better maturation. However, Oyesanya et. al.[317] note that collection of oocytes is related to the presence of a detectable velocity waveform, but not to the size of follicles.

Previous studies have involved the use of mean values for the PSV from many follicles[323], the maximum PSV from serial monitoring[258] and the PSV from individual follicles[319,324]. When mean values for follicular PSV[323] and maximum PSV from serial monitoring[258] were evaluated, no differences in values between conception and non-conception cycles was noted. However, when individual follicles, oocytes, and preimplantation embryos were studied, a significant difference in PSV was found in conception compared with non-conception cycles.

The studies that minimize the inter-follicle differences[258,323] yield no-difference results and those detailing individual follicles show significant results[319,324]. Thus, it is the individual follicles, not the cohort that determines successful outcome.

1.5 Angiogenesis

During embryo development, blood vessels differentiate from endothelial precursors by a process called vasculogenesis. In adults, further vessel development from pre-existing vasculature occurs by angiogenesis[325]. Physiological angiogenesis is an integral part of fetal development but occurs less commonly in adults, except during wound healing and in the female reproductive tract[326,327]. Pathological angiogenesis takes place in conditions such as solid tumour growth, chronic inflammatory disorders, endometriosis and diabetic retinopathy[328].

Angiogenesis can occur by a number of mechanisms and is influenced by a wide range of growth factors, receptors and inhibitors. Proposed mechanisms for the creation of new blood vessels from the existing vasculature are vascular sprouting, intussusception and vessel elongation.

1.5.1 VEGF and VEGFR

Vascular endothelial growth factor (VEGF) is a family of angiogenic growth factors characterized by their ability to promote vascular endothelial cell proliferation and increase vascular permeability[329]. VEGF has been found to induce fenestrations in endothelial cells of small venules and capillaries[330] as a crucial step in angiogenesis. This allows fibrinogen and other serum proteins to exit the capillary space and form an extravascular fibrin gel. This matrix then supports the ingrowth of endothelial cells and other elements to generate new vascularized stroma. VEGF also stimulates nitric oxide release from endothelial cells[331] and induces the release of prostacyclin by activating cytosolic phospholipase A_2, causing the release of arachidonic acid[332]. Both of these actions would be expected to promote vasodilatation.

Six members of the VEGF family have been described: VEGF-A through to VEGF-E and placental growth factor (PLGF). VEGF-A/vascular permeability factor (VEGF/VPF) is a basic, heparin-binding, homodimeric glycoprotein of 45 KD. Its gene has been localized to chromosome 6p21.3, composed of eight exons. VEGF-A through to VEGF–D can occur in a number of different isoforms due to alternative splicing. Five molecular forms of VEGF-A are produced in humans as a result of such alternative splicing of the gene transcript. Three of these, VEGF121, VEGF145 and VEGF165, are soluble isoforms, but the two larger forms, VEGF189 and VEGF206, are generally membrane-bound. These longer splice variants of VEGF-A also bind heparin strongly and are not as freely available within the tissues as the shorter splice variant.

VEGF165 is the predominant molecular form produced by a variety of normal and transformed cells. Transcripts encoding VEGF121 and VEGF189 are detected in the majority of cells and tissues expressing the VEGF gene. In contrast, VEGF206 is a rare form, and has been described only in the human fetal liver[333].

VEGF proteins exert their functions through a family of closely related receptor tyrosine kinases, called: VEGFR-1 / Flt 1 / fms-like-tyrosine kinase; VEGFR-2 / KDR / Flk-1 / kinase domain region; and VEGFR-3 / Flt-4[334].

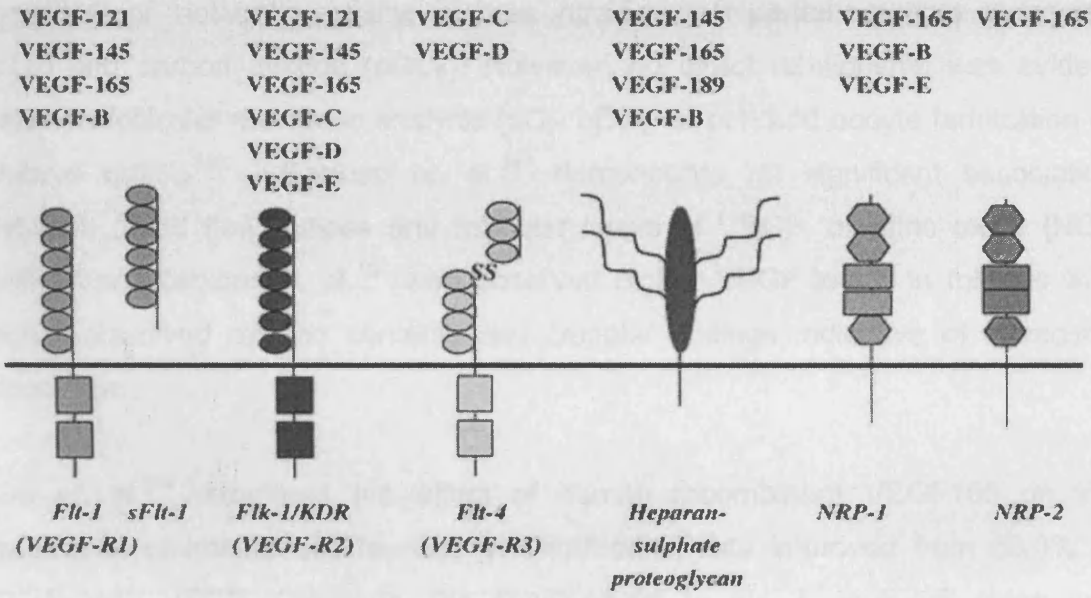
Each member of the VEGF family has specificities for different receptors with different angiogenic activities (Figure 1.2). VEGF-A mediates most of its angiogenic activities via VEGF-R2 but also binds to VEGF-R1. VEGF-B and PLGF only bind to VEGF-R1 and do not have strong angiogenic activity. VEGF-C and –D bind to VEGF-R2 and VEGF-R3 and have major roles in regulating lymphatic angiogenesis. VEGF-E is a protein encoded by a strain of the parapox virus and only binds to VEGF-R2. VEGF-A can also bind selectively to two neuropilins, NRP-1 (VEGF165) and NRP-2 (VEGF165 and VEGF145)[335].

It is proposed that VEGF-R1 and -R2 mediate different VEGF-A activities. The VEGF-R2 system is involved in endothelial cell formation, migration, and

proliferation. In contrast, the VEGF-R1 system is important for endothelial cell-to-cell interaction and vessel formation. Also described is a soluble form of VEGFR-1, which lacks the transmembrane and tyrosine-kinase domains essential to influence endothelial cell function. This soluble form of VEGFR-1 (sVEGFR) binds VEGF and prevents its action on VEGFR-1 and -2. It has been demonstrated in a rat model that sVEGFR-1 can cause corpus luteum failure, with secondary failure of endometrial decidualization[336].

Angiogenesis is tightly down-regulated in the normal state. Several proteins that inhibit angiogenesis, including thrombospondins[337,338], endostatin[339], angiostatin, endostatin, platelet factor 4 and transforming growth factor β[340] have been discovered.

Figure 1.2. VEGF and VEGR



1.5.1.1 VEGF-VEGFR and Fertility

Van Blerkom[341] demonstrates that both genetic and epigenetic factors are associated with the developmental competence of human oocytes and embryos created by IVF. The degree of perifollicular vascular expansion associated with increased rates of blood flow is developmentally important for the generation of a

normal follicle and competent oocyte. The findings from >1000 samples of follicular fluid show that oocytes from severely hypoxic follicles are associated with high frequencies of abnormalities in the organization of the chromosomes on the metaphase spindle that could lead to segregation disorders and catastrophic mosaicisms in the early embryo. Oocytes with cytoplasmic defects and cleavage stage embryos with multinucleated blastomeres are derived predominantly from severely hypoxic follicles[107]. The degree of vascular development was found to be follicle specific and differences between follicles might reflect their unique abilities to regulate angiogenic growth factor production by the follicle cells in response to hypoxia.

Doppler indices, which are markers of downstream impedance to blood flow, have a significant and negative correlation with the cleavage status of day 3 embryos[342]. Moreover, these indices of follicle-specific vascularity also correlated with measures of metabolic activity, such as intra-follicular partial pressure of oxygen (pO_2) and carbon dioxide (pCO_2). However, no direct relationship was evident between follicular metabolic analysis (pO_2, pCO_2, or pH) and oocyte fertilization or embryo quality[342]. Barroso et. al.[343] demonstrate no significant association between blood flow indices and follicular levels of VEGF, or nitric oxide (NO), unlike Van Blerkom et. al.[107] who observed higher VEGF levels in follicles with higher dissolved oxygen contents and Doppler findings indicative of increased blood flow.

Luo et. al.[344] examined the effect of human recombinant VEGF165 on the maturation of bovine oocytes and the fertilization rate improved from 63.0% to 82.3% with VEGF. Cleavage, the development to the 4- to 8-cell stage and blastocyst rates were also significantly higher in the VEGF group than the control group.

Follicular fluid VEGF concentrations were more than 10-fold greater than serum concentrations at the time of oocyte collection, thus implying that the peri-

ovulatory, luteinising follicle produces significant amounts of VEGF[345,346,347]. Doldi et. al.[348] found strikingly higher progesterone levels in the follicles of low responders compared with normal responders. Likewise, VEGF was higher in the mature follicles of low responders, consistent with more advanced luteinisation. The observation of positive correlation between follicular fluid VEGF and both follicular fluid and serum progesterone concentration was considered as an argument favouring the hypoxia hypothesis[345] because premature luteinisation may be an early manifestation of limited ovarian reserve or ovarian ageing[349,350]. This also gives credence to the hypothesis that elevated VEGF concentrations may reflect simply exuberant and/or early luteinisation near the time of hCG administration. Recent publications also report similarly diverging outcomes (Table 1.4).

The reconciliation of these diverging outcomes may be reached with the observation that VEGF levels are determined by multiple factors. If VEGF levels are induced by hypoxia then the corresponding oxygen levels would be lower than when VEGF is induced by non-hypoxic stimuli, such as inherent superiority to produce higher levels of VEGF at a given oxygen level.

Poor ovarian response to gonadotrophin stimulation is also proposed to be the result of inhibited VEGF bio-availability. Hypoxemia enhances VEGF expression[360] in maturing follicles and augments VEGFR-1 production in corresponding endothelial cells[359]. Alternative splicing of the VEGFR-1 mRNA to produce sVEGFR-1 significantly contributes to the regulation of VEGF activity[365]. Rising sVEGFR-1 production and secretion in the vicinity of maturing follicles is detrimental to further development. Thereby, excessive sVEGFR-1 production in ovarian blood vessels results in poor ovarian response due to diminished bio-active VEGF supply. High follicular VEGF production cannot overcome the blocking effects of sVEGFR-1. Concentrations of VEGF in follicles from women with poor ovarian response to stimulation may be consequently higher than in women with pronounced ovarian response to gonadotrophin stimuli.

Neulen et. al.[366] defined a surrogate indicator for biological activity of VEGF by VEGF:sVEGFR-1 ratio. Higher values of this ratio revealed an increasing availability of VEGF and this was associated with higher ovarian response to gonadotrophin therapy. A significant negative correlation between soluble VEGFR-1 concentrations and the number of collected oocytes was also detected.

Table 1.4. Studies on Serum and Follicular Fluid VEGF and Assisted Conception

	Serum VEGF	**FF VEGF**
Manau[347] Lee[345]	↔ Ovarian response	↑ Female age ↑ Total gonadotrophin dose
	↔ IVF outcome	↔ IVF outcome ↔ FF oestradiol, progesterone
Christenson and Stouffer[351]		↑ Duration of stimulation ↑ Total gonadotrophin dose
Artini[352] Enskog[353] Licht[354]	↑ Ovarian response ↑ Luteal progesterone	↑ Duration of stimulation ↑ Total gonadotrophin dose
Anasti[355]		↑ FF progesterone ↑ Serum LH
Doldi[356,348] Van Blerkom[107] Van Blerkom[357] Puchner[358]		↑ Ovarian response ↑ Follicles with high vascularization and oxygenation, ↑ Fertilization, pregnancy rates
Shweiki[359] Gerber[360]		↑ Follicular hypoxia
Friedman[49,361] Barrosso[343] Battaglia[362]		↓ Ovarian reserve ↓ Ovarian response ↓ Serum oestradiol ↓ Embryo quality ↓ Pregnancy rate
Quintana[363]		↓ Ovarian response ↓ Apoptotic granulosa cells
Benifla[364]		↔ IVF outcome ↔ Causes of subfertility ↔ Female age

↑: *Positive association*, ↓: *negative association*, ↔: *no association*, FF: *Follicular fluid*

1.6 Endometrial Receptivity

In humans, implantation is a process of initial apposition of maternal and embryonic epithelium, intrusion of embryonic cells between the maternal luminal epithelial cells, and invasion of placental cells deep into the endometrium. The process of implantation is inefficient and demands that minimum requirements be met. These depend on the synchronization of maternal and embryonic clocks. The maternal clock begins at ovulation when steroid production in the ovary switches from oestrogen to progesterone plus oestrogen, which causes the endometrium to differentiate such that it reaches a state of readiness for implantation approximately seven days after the LH peak, or 5.5 days after ovulation[367]. Implantation occurs in the mid-secretory phase of the menstrual cycle when the stroma is undifferentiated, but decidualization has occurred within three days of implantation [368,369].

In some cases, this sequential maturation of the endometrium and the related implantation process can be delayed, allowing some of the slowly developing embryos to implant and produce pregnancies[370]. However, the endometrium cannot retain its receptivity for an indefinite period[371]. Lenton et. al.[372], studying patterns of hCG secretion in natural pregnancies, suggest that the window of implantation in humans extends to post-ovulatory day 10, which is between days 19 and 24 of natural cycles. It is suggested that it might be preferential to perform ET when embryos are at a more advanced stage than uterine maturation, as noted in animal studies.

Pinopodes are smooth membrane projections from the luminal cell surface of the endometrium at the time of implantation[373,374]. In humans they have been observed at around day 20 of a natural cycle. Their entire life span does not exceed 48 hours and expression of fully developed pinopodes is limited to only one day. Pregnancy rates have been shown to improve with adjustment of the day of transfer so that a 6-day embryo would encounter an endometrium with fully developed pinopodes[375].

The standard assessment of endometrial maturation has been the histological dating of an endometrial biopsy[376]. Delayed endometrial maturation was more frequent in the non-pregnant group than the pregnant group[377]. However, there are major disadvantages in endometrial biopsies, such as their invasiveness and the negative impact on the implantation process. The timing and number of biopsies, methods used for chronological standardization and the extent of discrepancy required to define an endometrial biopsy as being 'out of phase' remain in contention[368]. Furthermore, assessments performed in a natural cycle may not be predictive of the quality of the endometrium in a subsequent stimulated cycle.

Serum oestradiol concentrations or oestradiol:progesterone ratios are also reported to be inaccurate in predicting the histological state of endometrial development, which can occur over a wide range of serum concentrations[378,379].

As an alternative, high-resolution transvaginal ultrasonography makes it possible to monitor histological changes in the endometrium indirectly through the analysis of endometrial growth and echogenicity. In general, sonographic parameters provide a high negative predictive value and sensitivity, but a limited positive predictive value and low specificity[288]. The reported low specificity in various studies, ranging between 9 and 35%, indicates that uterine receptivity is one among several different factors contributing to implantation.

1.6.1 Endometrial Thickness and Echo-pattern

Endometrial thickness is defined as the maximum distance between the echogenic interfaces of the myometrium measured in the central longitudinal axis of the uterine body. Endometrial pattern is defined as the type of relative echogenicity of the endometrium and adjacent myometrium. Endometrial pattern varies during the menstrual cycle[380]. Smith et. al.[381] describes four different patterns and Gonen and Casper[382] describe a simplified version with three types; Type A representing an entirely homogeneous, hyperechogenic pattern without a central echogenic line; Type B an intermediate iso-echogenic pattern with the same reflectivity as the

surrounding myometrium and a non-prominent or absent central echogenic line; and Type C a multilayered 'triple-line' endometrium consisting of a prominent outer and central hyperechogenic line and inner hypo-echogenic or black region. Sher et. al.[383] propose a more concise classification with two grades: a non-multilayered, homogeneous, hyperechogenic or iso-echogenic endometrium compared with the myometrium, and a multilayered triple-line pattern with an outer peripheral layer of denser echogenicity with a central sonolucent area. The central echogenic line represents the uterine cavity and the outer lines the basal layer of the endometrium or the interface between the endometrium and the myometrium. The hypo-echogenic regions between the two outer lines and the central line represent the functional layer of the endometrium[384].

During the follicular phase in natural cycles, the endometrium is hypo- or iso-echogenic compared with the surrounding myometrium, while the endometrium to myometrium interface and the uterine cavity appear hyperechogenic[382,385] as a reflection of the glandular straightness, reduced glandular secretion, and reduced stromal oedema. After ovulation, endometrial echogenicity increases progressively, with hyperechogenic changes developing from the base toward the surface of the endometrium[386], parallel with the rising progesterone levels[387]. It is reported that in ovarian stimulation cycles, premature exposure of the endometrium to progesterone during the follicular phase leads to a faster progression of endometrial echogenicity during the early luteal phase[387]. Stromal oedema, glandular coiling and secretion are thought to be the key events responsible for hyperechogenic signals[386].

1.6.1.1 Endometrial Thickness

Endometrial thickness as a predictor of IVF-ET outcome has been investigated by numerous studies with variable results. While some study groups found a significant correlation between thickness of the endometrium and pregnancy rate[383,388,389,390,391,392,393,394], others reported no such relationship[256,257,277, 281,395,396, 397,398,399,400,401,402,403,404,405]. It is reported that there is an extensive overlap in the

ranges of endometrial thickness present in pregnant and non-pregnant cycles[406]. A pooled comparison of the published data on 2665 cycles reports that ranges of mean endometrial thickness for conception and non-conception cycles are almost the same (8.6 to 11.8 and 8.6 to 11.9 respectively)[288].

These opposing conclusions may in part be due to the different techniques used, such as vaginal vs abdominal ultrasonography, different ovarian stimulation protocols[293], measurement errors in obtaining a standard sagittal view of the uterus, or marked ovarian enlargement distorting the endometrial outline[407].

On the basis of the strong correlation between uterine dimension and endometrial thickness, Strohmer et. al.[408] suggests that endometrium thickness is determined by individual uterine architecture and so is not predictive of the likelihood of implantation. Similarly, no correlation was found between implantation and the mean cross-sectional area of the endometrium[407]. Endometrial thickness, as a proxy measure of endometrial growth, is reported to be unrelated to endometrial pattern on the day of hCG injection[389,390,395].

The published results on natural cycle FTER report no differences in the endometrial thickness between the pregnant and non-pregnant groups[256,409,410]. In hormone replacement cycles for FTER and oocyte donation, results are more variable. While some published non-significant differences in endometrial thickness between the pregnant and non-pregnant groups[256,277,409], Abdalla et. al.[391] (10.2±2.63 vs 8.6±3.49mm), Shapiro et. al.[412], and Alam et. al.[411] (10.5±3.5 vs 9.6±4.2mm) report significant differences.

Schild et. al.[281] found that mean values for endometrial volume assessed by three-dimensional ultrasonography, are non-significantly higher in conception vs non-conception cycles. No conception was observed below an endometrial thickness of 6.9 mm and volume of 1.59 ml on the day of oocyte collection. Raga et. al.[413] also found that a minimum volume of 2 ml is a prerequisite for a receptive endometrium

and that no pregnancy was achieved when endometrial volume measured <1.2 ml. Beyond an endometrial volume of 2 ml, no relationship was apparent in terms of endometrial receptivity.

Various values of endometrial thickness between 6 and 10 mm have been proposed as discriminatory between conception and non-conception cycles, albeit with a low specificity and low positive predictive value. However, recent evidence indicates that embryonic implantation is possible even when endometrial thickness is <4 mm[394,399]. The main advantage of ultrasonographic assessment of the endometrial thickness is given as its high negative predictive value in cases where minimal endometrial thickness was not reached.

1.6.1.2 Endometrial pattern

Some investigators have ascertained that endometrial echogenic patterns in the late follicular phase predict IVF-ET outcome[256,257,277,382,383,390,392,393,396,404,405,414,415,416,417,418,419]. A triple-line pattern appears to be the sonographic parameter that mostly reflects endometrial receptivity as it is associated more frequently, but not exclusively, with conception cycles. On the contrary, others have failed to find a relation between endometrial echogenicity and implantation rates[397,417,420,421].

The method of endometrial preparation was found to have no specific influence upon the endometrial pattern because no significant differences were noted between conceptional and non-conceptional cycles of natural and HRT cycles[409] or between natural IVF and stimulated IVF cycles[404,422]. However, among natural cycles for FTER, ovarian stimulation cycles with fresh ET and HRT cycles for oocyte donation, it was only in HRT cycles for oocyte donation that a better positive predictive (47.8%) and specificity (42.8%) values were observed[412].

It is proposed that more advanced hyperechogenic transformation of the endometrium at the time of hCG administration is associated with lower implantation and pregnancy rates in IVF[397,423]. The mechanism to explain this

inverse relation is proposed to be an alteration of the endometrial receptivity, which results from accelerated secretory transformation of the endometrium. Reports have shown that echogenicity status reflects the degree of histological development of the endometrium[385,386,424].

Sher et. al.[383] observed a higher prevalence of poor endometrial grade in women aged >40 years, as well as in women with uterine pathology. No relationship was demonstrated between the mid-luteal phase serum progesterone values and type of echo pattern[425]. Check et. al.[425] also report that in FTER cycles pre-ovulatory LH concentrations were highest in the group with the homogeneous hyperechogenic pattern, which has the best pregnancy prognosis. Ohno and Fujimoto[426] found no relationships between endometrial appearance, endometrial steroid receptors and steroid hormone concentrations in serum.

Positive correlation detected between increasing maturity of the ultrasonographic endometrial pattern and serum oestradiol concentrations was not supported by later studies[390]. No correlation was noted between serum oestradiol concentrations and endometrial thickness or pattern during both natural and ovarian stimulation cycles[382,388,397]. Time-dependent rather than concentration-dependent thickening of the endometrium is reported throughout the follicular phase of the menstrual cycle[427,428]. It is hypothesized that supraphysiological E_2 levels induced by ovarian stimulation accelerate neither the pace nor the magnitude of endometrial development beyond values triggered by physiologic concentrations of oestrogens[427]. In contrast, Ueno et. al.[404] shows that mean endometrial thickness is significantly thinner in natural cycles compared with cycles of ovarian stimulation on the day of the LH surge, (8.9 ± 8.0 vs 10.6 ± 2.5 respectively).

1.7 Frozen-Thawed Embryo Replacement

Ovarian stimulation protocols using GnRH analogues and gonadotrophins yield large numbers of oocytes. With improved culture methods, a higher number of good-quality embryos have become available for transfer. Attempts to reduce the

incidence of multifetal pregnancies by transferring no more than two or three embryos, has further resulted in an increased number of embryos not used for immediate transfer after oocyte collection. Cryopreservation of supernumerary embryos at the pronuclear, multicellular (four-eight blastomeres) or blastocyst stage and transfer after thawing, increases the overall pregnancy rate in single oocyte collection procedures.

Since the first report[429] of successful FTER, the cryopreservation of embryos has been an important supplementary procedure in the treatment of subfertility. For those couples who have embryos cryopreserved, frozen-thawed ET provides an additional chance of pregnancy [430,431]. Embryo cryopreservation has also provided additional clinical safety in the presence of ovarian hyper-stimulation and contributed to lowering the probability of multiple conception by reducing the need to transfer multiple fresh embryos[432]. However, FTER is not free from the probability of multiple conception, although the implantation rate of frozen-thawed embryos is usually lower than that of fresh embryos. The multiple conception rate following FTER is reported by the Australian and New Zealand registry to be 13%[433]; 15 times higher than in spontaneous conceptions.

The overall implantation rate following FTER ranges from 8–11%[409,434,435] but higher figures are also published (18%) for frozen-thawed embryos following ICSI[436]. After FTER the reported pregnancy rate is 7.7–27%[437,438].

A crucial factor for implantation in FTER is the synchronization of endometrial maturation and embryo development. For women who menstruate regularly, such synchronization may be achieved successfully after spontaneous ovulation[439,440,441]. For women with functioning ovaries but with anovulatory or irregular cycles, FTER should take place only after adequate endometrial preparation, which can be achieved by ovulation induction with either clomiphene citrate or gonadotropins[442,443]. Navot et. al.[444] reports that adequate endometrial maturation can be achieved by exogenous hormone administration to women with

ovarian failure. Subsequently it is reported that women with functioning ovaries can also have an artificially prepared endometrium using exogenous steroids after pituitary suppression by GnRH-a [440,445,446,447,448]. A receptive endometrium is reported after priming with oestradiol for two weeks (or more) and progesterone added to the regimen 3–4 days prior to ET [449].

For simplicity, these regimens can be subdivided into two main subcategories: those with GnRH analogue[447,448] and those without[450,451,452,453,454]. In both subcategories hormones can be administered either in a fixed dose regimen throughout the protocol[412,455,456,457,458,459,460] or in a sequential dose regimen to mimic the natural menstrual cycle[277,456, 459,461,462,463, 464].

17β-estradiol can be given either orally (in valerate or micronized form), vaginally, transdermally, or by subcutaneous implant[465,466] in order to bypass the gastrointestinal tract, thus avoiding first pass metabolism. Progesterone can be administered by intramuscular injections, vaginally or sublingually[467]. Oral micronized progesterone is only effective at higher doses; it is absorbed variably and metabolized by the liver (first pass effect). Due to unphysiological metabolites this route has a high rate of unwanted side effects. The IM injection of progesterone is safe and effective, but painful and inconvenient for the patient, since involvement of another party is necessary. Vaginal suppositories with progesterone are effective in ET cycles but whether the vaginal route results in better secretory endometrial transformation remains controversial. This stems from the fact that vaginally administered progesterone results in adequate secretory endometrial transformation, despite serum progesterone values lower than those observed after IM administration, even if they are lower than those observed during the luteal phase of the natural cycle. This discrepancy is indicative of the first uterine-pass effect and of better bioavailability of progesterone in the uterus[468].

1.7.1 HRT Cycles with and without Down-regulation

Although traditionally the use of GnRH-a suppression is considered essential for adequate endometrial hormonal modulation[448], several studies question its necessity for controlled endometrial preparation in both FTER cycles[450,451,453,354,469] and for recipients of oocyte donation who have functioning ovaries[465,470]. These studies suggest that avoiding GnRH agonist makes the procedure simpler, quicker and cheaper. It is also emphasised that when the pituitary is not suppressed by using a GnRH agonist, oestradiol treatment should be initiated in the early follicular phase (on days 1 or 2). Although initial follicular activity may be present, with this approach spontaneous ovulation is successfully inhibited. Starting oestradiol treatment after cycle day 3 might lead to an increased incidence of LH surge and luteinisation of the endometrium[471]. The starting dose of oestradiol does not seem to be as important. Simon et. al.[451,452] chose a high fixed dose of micronized oestradiol (6 mg/d). Pattinson et. al.[454] used a lower fixed dose of micronized oestradiol (2 mg/d) starting from cycle days 2 to 5, and report a cancellation rate of 7.4%. A step-up protocol using 100 µg to 400 µg of oestradiol administered by transdermal patches has been adequate for inhibition of ovulation and endometrial preparation[450,453].

Krasnow et. al.[472] compared the effects of oral micronized E_2 with transdermal E_2 on endometrial receptivity in women having oocyte donation. It is concluded that supraphysiological serum E_2 levels associated with oral micronized E_2 may have a deleterious impact on endometrial receptivity. The development of more physiologic hormone replacement protocols is proposed, to enhance endometrial receptivity and lead to improved clinical pregnancy rates.

1.7.1.1 Duration of HRT

The sole use of exogenous oestrogen and progesterone was shown to prime optimal endometrial receptivity in women with failed or absent ovaries. Luteal E_2 levels do not impact on endometrial morphology. After sufficient priming by E_2 only, progesterone alone appears instrumental in triggering the proper sequence of

secretory transformation in glands and stroma with no clinical role for the E_2: progesterone ratio. Secretory changes in endometrial glands are best seen between the day 4 and day 6 of progesterone administration and predecidual changes of the endometrial stroma are apparent from day 10 of progesterone exposure.

In 192 oocyte donation cycles, Prapas et. al.[473] show that the window for ET is dependent on duration of treatment with progesterone, beginning approximately 48 hours after starting progesterone administration and lasting for approximately four days. The optimum phase for transferring embryos at the four- to eight-cell stage corresponds to cycle days 18 and 19. Navot et. al.[474] studied the flexibility of E_2 priming by analyzing the consequences of varying the duration of the E_2-only phase in the HRT cycles of donor oocyte recipients. It is reported that the E_2-only phase could last from five days to six weeks without altering the quality of progesterone induced endometrial receptivity to the embryo.

Younis et. al.[475] investigated the limits of a prolonged endometrial preparation on women with ovarian failure. Three study groups were treated with oral oestradiol and oestriol 4 mg/day for 21, 28, and 35 days respectively then intramuscular progesterone 50 mg/day was added for a further seven days. All late follicular biopsies showed a normal proliferative endometrium. The mid-luteal biopsy showed a secretory endometrium with no significant glandular-stromal disparity. It is concluded that an artificial prolonged follicular phase does not seem to adversely affect the endometrial developmental capacity.

Younis et. al.[460] further investigated whether such manipulations of endometrial stimulation could influence the pregnancy rate. It seems that for optimal results in an oocyte donation programme, oestrogen stimulation should be kept at between 12 and 19 days. Likewise, Michalas et. al.[470] observes that transfer of embryos between days 17 and 19 of the recipient's cycle provided the best possible clinical outcome.

However, Yaron et. al.[476] show that the endometrium is tolerant to varying durations of E_2 stimulation with regard to pregnancy rates in oocyte donation cycles. Similarly, successful implantation was observed even after 100 days of unopposed oestradiol valerate administration[448]. However, because of the high incidence (>44%) of break-through bleeding after nine weeks, it is advised to stop oestrogen treatment at this point. In contrast, a proliferative phase of <10 days is found to be related to a higher miscarriage rate[474,477].

1.7.2 HRT Cycle vs Natural Cycle

Although FTER in a natural cycle is less expensive and may be more acceptable, as it does not require exogenous hormones, ET in a hormone replacement cycle is more flexible and better controlled. The timing of ET in a natural cycle requires accurate determination of ovulation but daily hormone tests and ultrasonographic monitoring can be inconvenient for the patient. On the other hand, hormone replacement cycles, do not require intense monitoring. They give the flexibility of timing the transfer date with lower cancellation rates when compared with the commonly cited 6% cancellation rate in natural cycles[478]. The disadvantages of HRT regimens include the need to continue the exogenous hormones throughout the first trimester.

Some authors report higher pregnancy rates in hormonally controlled cycles than in natural cycles, but mostly in small groups of cases[440,445], while others using large semi-randomized or retrospective studies report similar outcome in both types of cycles[478,479]. The available data from these studies suffers from the methodological problem of comparing two unidentical groups in their response to two different therapeutic regimens (Table 1.5).

Dor et. al.[480] compared three different methods of cycle preparation prior to FTER. In natural cycles the pregnancy rate was 16.1%, in cycles with an ovarian stimulation protocol 11.4% and in cycles with oestrogen and progesterone substitutional therapy 9.5%. The differences were not significant. In the natural

cycles the implantation rate was 29.2% when the endometrium was synchronized with embryo development or advanced 32 hours beyond embryo development.

In a comparative study Imthurn et. al.[481] investigated 31 natural cycles and nine cycles programmed with a short-term stimulation protocol as preparation for FTER. In the natural cycle group the cancellation rate was 48% and two clinical pregnancies were established (13% per replacement). In the stimulated group no cycles were cancelled and eight replacements were performed, resulting in three clinical pregnancies (38% per replacement) and one biochemical pregnancy.

In 236 women undergoing 381 consecutive FTER cycles, Tanos et. al.[482] compared the efficacy of three protocols of endometrial preparation: spontaneous cycles, HRT preparation, and ovarian stimulation. No statistically significant difference was found in implantation rates per ET (5.6%, 5.6%, 4.6% respectively) and clinical pregnancy rates per cycle (16.9%, 16.5%, 15.6% respectively).

1.7.3 Factors Affecting the Treatment Outcome of Frozen-Thawed Embryo Replacement Cycles

High-quality embryos are known to sustain less cryoinjury during cryopreservation when compared with those of moderate and poor quality. When cryopreservation was performed at the one-, two-, and three-day stages of embryo development, pregnancies were similarly achieved with most of the embryos at all cell stages. Asynchronism of endometrial growth to the cell stage does not appear to reduce pregnancy rates. Kondo et. al.[483] concludes that embryo quality evaluated morphologically is the most important clinical factor for successful implantation of frozen-thawed embryos.

In a retrospective study Wang et. al.[484] analysed 3570 FTER cycles in 1438 couples, to evaluate the clinical circumstances that influence the potential for embryo implantation. The characteristics associated with a more favourable implantation rate were the success of the previous fresh ET cycle, female age <40

years and non-tubal factor aetiology of subfertility. For women in the poor prognosis group with a single embryo transferred the pregnancy rate was <10%. In the good prognosis group, with two embryos transferred the pregnancy rate was >20% and the probability of multiple pregnancy 15–29%. In the majority of the women, with three embryos transferred the probability of pregnancy was no higher than for two-ET but the probability of multiple conception was increased.

To determine the outcome of FTER, Loh and Leong[485] studied endometrial preparation, number of embryos transferred, female age at embryo freezing and endometrial thickness, with the most important clinical factor appearing to be the type of endometrial preparation. The natural cycle pregnancy rate was almost twice that of the hormone replacement cycles. Other factors that were suggestive of success were younger female age at embryo freezing, transfer of at least two embryos and endometrial thickness ≥11 mm.

Table 1.5. Frozen-Thawed Embryo Replacement Cycles: HRT vs NC

Study	Number of Cases	HRT Protocol	Cycle Monitor	Outcome in HRT cycle	Outcome in Natural Cycle	Cancellation Rate	Sig.
Cameron[486]				PR: 24%	PR: 14%		No
Schmidt[445] Prospective	12 women with HRT 26 women with NC	GnRHa Transdermal E_2 Sequential dose PV P_4	TVS Urinary LH Serum LH, P_4	PR: 17%	PR: 8%	7/33 in NC	Yes
de Ziegler[487]	65 cycles with GnRHa + HRT 18 cycles with HRT 53 cycles with NC			Without GnRHa PR of embryos originating from donated oocytes: 33% With GnRHa PR: 6%	PR: 11%		
Dor[480]				PR: 9.5%	PR: 16.1%		No
Muasher[440] Retrospective	22 cycles with HRT 144 cycles with NC	GnRHa Transdermal E_2 Sequential dose IM P_4		CPR: 36% IR: 18%	CPR: 21% IR: 10%		Yes
Sathanandan[478] Retrospective	84 patients with HRT 78 patients with NC	GnRHa E_2 valerate Sequential dose IM P_4	TVS Serum LH, P_4, E_2	CPR: 20%	CPR: 20%	No cancellation in HRT 4/78 in NC	No
Pattinson[454] Prospective	54 cycles with HRT 165 cycles with NC	No GnRHa Micronized E_2 Fixed dose of 2mg/day from day 2-5 start PV P_4	TVS Serum LH, P_4	PR: 20% OPR: 12%	PR: 14.4% OPR: 12.2%	15.8% in NC 7.4% in HRT	No
Schalkoff[488] Prospective	185 cycles with NC		TVS Urinary LH		PR: 27.6%		

Study	Number of Cases	HRT Protocol	Cycle Monitor	Outcome in HRT cycle	Outcome in Natural Cycle	Cancellation Rate	Sig.
Al-Shawaf[409] Prospective	77 cycles with NC 75 cycles with HRT	GnRHa E_2 valerate Fixed dose of 4-6mg/day PV P_4	TVS Urinary LH	PR: 25% IR: 10.6	PR: 26% IR: 10.3%		No
Queenan[479] Prospective	230 with HRT 398 cycle with NC	GnRHa Transdermal E_2 Sequential dose IM P_4	TVS Serum LH, P_4, E_2	PR: 30% IR: 10.3%	PR: 28% IR: 11.9%		No
Tanos[482]				PR: 16.5% IR: 5.6%	PR: 16.9% IR: 5.6%		No
Loh and Leong[485]				PR: Half of NC	PR: 17.7%		

Sig: Significance, PR: Pregnancy rate, CPR: Clinical pregnancy rate, IR: Implantation rate, OPR: Ongoing pregnancy rate, NC: Natural cycle for frozen-thawed embryo replacement, HRT: Hormone replacement therapy for frozen-thawed embryo replacement, P_4: Progesterone, E_2: Oestradiol, TVS: Transvaginal ultrasonography, PV: Intravaginal, IM: Intramuscular

2 Material and Methods

2.1 Factors Affecting the Outcome of IVF Treatment

From the computerised database of the Lister Hospital, Assisted Reproduction Unit, clinical and embryologic data on fresh IVF treatment cycles between January 1994 and July 2001 were identified. Overall success of the IVF treatment showed noticeable improvement during this long period. However, as distribution of the prognostic characteristics was an intrinsic feature of the patient population, it was not anticipated that improvement in the outcome would affect the analysis. Treatment cycles involving oocyte or embryo donation, and FTER were excluded. Raw data on the demographic characteristics of the couples, diagnostic characteristics of subfertility, response parameters to ovarian stimulation, features of oocyte maturity and embryo development, as well as details of the ET procedure and treatment outcome had been logged into a FoxPro database in the form of separate files. To establish a more flexible data-management platform, each file was converted into SPSS format and scrutinised for reliability. Separate files were then merged into a master file using the cycle code of each treatment as the unique key variable. Consequently, clinical and embryologic variables of the 12,332 fresh IVF cycles were combined, making it possible to perform multiple analyses.

The aim was to identify the prognostic significance of variables in the outcome of assisted conception in terms of ongoing pregnancy and multiple pregnancy; and using these variables, to define the characteristics of good prognostic couples whose pregnancy chances were independent of the number of embryos transferred.

In this analysis, it was acknowledged that direct assessment of the factors immediately engaged in the complex biological processes of gamete production, fertilisation, and implantation may not always have been possible; because either which factors to measure or how to measure them were not known. Instead, it was proposed that surrogate clinical markers, which are easier to observe, measure and compare be identified to reflect the summative effect of the decisive factors of the outcome. It was also proposed that clinical

variables presenting the highest level of association with the outcome must have surrogated the most critical steps of the actual biological processes leading to the observed outcome. Despite having statistical significance in univariate analysis, some clinical variables surrogating the less critical aspects of the biological processes may lose their prognostic power when assessed with the other variables in multivariate analysis.

For this reason, firstly a univariate analysis was performed using clinical and embryological parameters as independent variables for ongoing pregnancy. Variables found to be significant were further evaluated in the multivariate analyses to identify the independent predictors of the treatment outcome with the highest level of impact. The analysis is based on 12,332 treatment cycles; however, the whole data were not available for each variable. A case with a missing value for either the dependent or the independent variable for a given analysis was not used in that analysis. Hence the number of cases included in the univariate and multivariate analyses may be different for each variable.

Secondly, prognostic significance of the number of embryos transferred was evaluated for the prediction of ongoing pregnancy with consideration of the quality of the embryos transferred, quality of the un-transferred sister embryos, and quality of the embryo cohort as a whole.

For practical reasons, embryo quality may not always have been available for decision making. Hence, thirdly, a series of receiver operating characteristic (ROC) curve analyses were employed for the 'short listed' prognostic markers for the implantation potential of embryos (female age, duration of subfertility, and the number of cleavage stage embryos created). The cut-off points of these prognostic variables obtained from the ROC curve analyses were then applied into the whole study population to divide it into subgroups. The aim was to achieve different subgroups that were individually homogenous for their prognostic characteristics.

Finally, in each of these subgroups defined by ROC curve analyses, prognostic significance of the number of embryos transferred was evaluated for the prediction of ongoing pregnancy.

Results were presented in the sequential order of events experienced during a typical IVF cycle: history taking for the demographic variables of the patients, ovarian stimulation (section 3.1); oocyte collection (section 3.2); fertilisation, development of the individual sister embryos and developmental quality of the embryo cohort as a whole (section 3.3, 3.4); quality of the transferred embryos and ET procedure (section 3.5, 3.6). The prognostic significance of the number of embryos transferred was presented in section 3.7 and 3.8. A similar analytic approach was employed for the assessment of the multiplicity of implantation in section 3.9. Clinical variables affecting the oocyte and embryo quality were also appraised. Oestradiol and FSH concentrations in serum were expressed in nanomole / litre (nmol/l) and international unit / litre (IU/l) respectively.

2.2 Low-Dose Aspirin Co-Treatment in Patients Undergoing IVF Treatment

A prospective, randomised, placebo-controlled, and double-blind clinical trial was conducted to evaluate the prognostic significance of tissue perfusion and its pharmacological manipulation with low-dose aspirin in the outcome of assisted conception. Utero-ovarian perfusion was assessed by Doppler ultrasonography. VEGF-VEGFR concentrations in serum and follicular fluid were also studied.

2.2.1 Patient Selection, Randomisation Process and Study Design

In the subfertile couples, female partners who were younger than 40 years and undergoing IVF treatment in the Aberdeen Fertility Centre, were invited to participate in the study. No limitations were applied on the grounds of type (primary or secondary), duration or aetiology of subfertility, or on the number and outcome of previous fertility treatments. Excluded from the study were women with contraindications to the use of non-steroidal anti-inflammatory drugs, or any current or previous significant systemic disease with the possibility of recurrence or sequelae.

Randomization was performed at the initial visit by drawing a sealed opaque envelope containing instructions on the individual patient treatment for either tablet 'A' or 'B'. Treatments were randomly assigned to the envelopes by using a random number generator in the Research Pharmacy Department. The bottling of the aspirin and matching placebo tablets was also performed by the Research Pharmacy Department. All patients and clinical staff were blinded to the type of treatment. The randomisation code was made available to the researchers only after the last recruited couple had completed their treatment.

A 10 ml blood sample was taken for VEGF and VEGFR concentrations as well as full blood count and baseline hormonal profile at the early follicular phase of the natural cycle. In those women with regular ovarian cycle, controlled ovarian stimulation was initiated with GnRH analogue in the mid-luteal phase of the previous cycle. Down-regulation was confirmed by serum oestradiol level and ultrasonography. Gonadotrophin was given in the form of recombinant FSH as per unit protocol. Once target follicular growth was achieved, 10,000 IU of hCG was administered intramuscularly. Luteal support was given as vaginal progesterone. At the mid-luteal phase when the GnRH analogue treatment was initiated, aspirin tablets at a dose of 75 mg twice daily and placebo tablets at a dose of one tablet twice daily were commenced and continued throughout the treatment. In the case of established pregnancy, aspirin and placebo tablets were continued until the end of the first trimester. Doppler assessments were performed on day 1 and on the last day of the ovarian stimulation before hCG injection. On the day of oocyte collection blood and follicular fluid samples were obtained for VEGF-VEGFR assessment.

Demographic characteristics of the couples and clinical and embryological variables of the *in vitro* fertilisation cycles were recorded. Clinical pregnancy, diagnosed by serum beta-hCG levels on day 15 after ET and confirmed by ultrasonography at the seventh week of pregnancy, was documented as the primary outcome measure. Ongoing pregnancy was defined as pregnancy beyond the first trimester. Biochemical pregnancy was defined as a significant but transient increase in βhCG levels (>10 mU/mL). The implantation rate was defined as a gestational sac per embryo transferred, visualised by ultrasound

three weeks after ET. 'No-ET' refers to the treatment cycles cancelled before the embryo transfer. 'No-ongoing pregnancy' refers to the treatment cycles with the outcome of no pregnancy, biochemical pregnancy, miscarriage during the first trimester, and ectopic pregnancy.

The clinical study was powered to evaluate the null hypothesis that 'low-dose aspirin co-treatment does not alter the clinical pregnancy rates in IVF treatment'. The inclusion of 310 patients in each arm of the study gave an 80% chance of detecting an improvement in the clinical pregnancy rates from 20% with no aspirin treatment to 30% with aspirin treatment at a 5% level of statistical significance. With an anticipated 10% dropout rate, recruitment of 680 couples was planned. For uterine artery Doppler analysis, 32 patients would provide 95% power at the 5% level of significance to detect a minimum difference of 0.71 (standard deviation: 0.54) in the mean uterine artery PI values between clinical pregnancy and no clinical pregnancy.

2.2.2 Protocol for Ovarian Stimulation

The Day 21-long protocol was the most commonly used regimen for ovarian stimulation in our department. Either buserelin acetate (Suprecur®; Hoechst Marion Roussel Ltd, Uxbridge, UK) 500 μg administered once daily as S.C. injections or nafarelin acetate (Synarel®; Searle, High Wycombe, UK) 400 μg administered twice daily as nasal spray was utilised for pituitary down-regulation.

The first ultrasound scan was scheduled usually at around 14 days after the initiation of the GnRH analogue treatment. This scan was to exclude the development of follicular cysts under the effect of GnRH analogue. Following the sonographic and biochemical confirmation of pituitary down-regulation (serum oestradiol concentrations of <0.1 nanomole/litre and the absence of follicular activity), ovarian stimulation was initiated with recombinant follicle stimulating hormone, follitropin beta (Puregon®; N.V. Organon, Postbus, the Netherlands; Gonal F, Serono, Italy), once a day as S.C injections. Follicular cycts >10mm were aspirated through tansvaginal route under ultrasonographic guidance before the initiation of ovarian stimulation.

On day 8 of the ovarian stimulation, the oestradiol concentration was measured. If the level was ≤0.1 nanomole/litre (nmol/l), the gonadotrophin dose (Puregon or Gonal F) was increased. If the level was ≥3 nmol/l the gonadotrophin dose was reduced and follicular growth was assessed by scan on day 9. If the oestradiol concentration was between 0.1 and 3 nmol/l the starting dose was maintained and the second pelvic ultrasound was scheduled for day 10 of the ovarian stimulation to assess the follicular response. Further scans were arranged accordingly. When the final scan revealed at least three follicles ≥17 mm, human chorionic gonadotrophin (hCG, Profasi®; Serono Pharmaceuticals Ltd, Middlesex, UK) 10,000 units was administered.

2.2.3 Oocyte Collection

Transvaginal oocyte collection under ultrasound guidance was performed as an outpatient procedure, 36 hours after hCG injection, using a double lumen oocyte harvesting needle. If no oocyte was obtained in the original aspirate the follicles were flushed up to three times. All patients received a preliminary I.V. loading dose of 4 mg midazolam (Hypnovel; Roche Products Ltd., Welwyn Garden City, UK) and 25 mg fentanyl (Sublimaze; Janssen-Cilag Ltd., High Wycombe, Bucks, UK). Maintenance bolus doses were then administered by the clinician performing the oocyte collection. Monitoring included a non-invasive blood pressure device, Dinamap (Criticicon Ltd., Bracknell, Berks, UK) and a pulse-oximeter. Blood pressure, heart rate and oxygen saturation were recorded at 5 min intervals during the procedure. The system used for oocyte grading is given below (Table 2.1).

Table 2.1: Oocyte Maturity

Oocyte	Corona cells	Cumulus	Germinal vesicle	Polar body
Immature	Tightly apposed	Tightly packed	Seen	Not extruded
Borderline mature	Radiating	Expanding	No longer seen	Extruded
Mature	Fully radiating	Expanded	No longer seen	Extruded
Post-mature	Little, dissociating	Dispersed	No longer seen	Extruded

2.2.4 Embryo Grading and Transfer

The system used for embryo grading is given below (Table 2.2). To complete the grading, the number of cells present was included as the 'stage'.

Table 2.2: Embryo Grading

Grade 1	Equal size of cells	No or few fragments
Grade 2	Unequal size of cells	No fragments
Grade 3	Equal size of cells	Lots of fragments
Grade 4	Unequal size of cells	Few fragments
Grade 5	Unequal size of cells	Lots of fragments
Grade 6	Undivided embryo	

A maximum of two embryos were transferred on day 2 after fertilisation. Because of the risk of ovarian hyperstimulation syndrome, fresh ET was not performed if serum E_2 concentration was ≥15 nmol/l on the day of hCG administration, if ≥30 follicles were aspirated, or if ≥20 oocytes were collected. For these patients all embryos were electively cryopreserved and transferred in subsequent cycles.

For the ET procedure, patients were in lithotomy position with a slight head down tilt. The cervix was exposed with a bivalve speculum. The vaginal walls and cervix were cleaned with warmed normal saline solution and finally with embryo culture medium. Any cervical mucus was removed gently with a moist swab. Grasping of the cervix was performed only when difficulty in introducing the catheter was encountered.

K-Jets-6019-SIVF catheters (Cook IVF, Queensland, Australia) were used for ET. The outer catheter has a pre-shaped curve to accommodate the natural flexion of the cervical canal and the uterus. The rounded bulb tip is designed to tract through the cervical canal. The catheter has two depth markers on the shaft at 4 and 5 cm from the tip. The mark at the external cervical os would correspond to the length of the cervical canal. The inner transfer catheter is a soft small diameter catheter with a rounded bullet tip. The proximal shaft is stiffened with an external cannula.

Drawing up of the embryos into the inner catheter was done with the three-drop procedure in which the embryos were separated by a 20 µl bubble of air. The total volume of medium was about 3 x 0.02 ml. Once the embryos were loaded, the inner transfer catheter was assembled into the outer catheter so that its tip would be 1cm inside the tip of the outer catheter. The catheter was then introduced into the cervical canal. When the internal os was just passed the inner catheter was advanced 2 cm into the uterine cavity. The transfer volume was then gently expelled by pushing the plunger of the attached syringe. The catheter was left in the cavity for 10 second (sec) and withdrawn slowly as a single unit. The two instruments were flushed with medium under a stereomicroscope to ensure that the embryos had been released into the uterine cavity.

Details of the transfer procedure were recorded, including duration, bleeding, mucus and excessive manipulation. The degree of difficulty of each transfer procedure was judged subjectively by the clinician on a scale of 1 to 4: 1 = very easy, when the catheter had passed effortlessly into the uterine cavity; 2 = easy, when the catheter required some manipulation and then passed freely; 3 = some difficulty, when catheter manipulation plus a tenaculum were required; 4 = very difficult, when multiple catheter changes and cervical dilatation plus a tenaculum were required.

At the end of the procedure, patients were permitted to empty their bladder 10–15 min after ET and they then rested for one hour in the recovery room. Thereafter, patients were discharged and advised to resume their usual activities. Following ET, progesterone pessaries (Cyclogest®; LD Collins&Co Ltd, London, UK) 400 mg once daily were given as luteal support.

2.2.5 Colour and Pulsed Doppler Measurement

Transvaginal colour and pulsed Doppler measurements were performed on the day that pituitary suppression was confirmed and on the day prior to hCG injection (day of last scan). To minimise the effect of circadian rhythm over the uterine blood flow[269], Doppler assessments were performed at the same time of day for each patient. Before being scanned the patients rested for at least 15

min and completely emptied their bladder to minimise any external effects on the pelvic blood flow[489]. All scans were performed by a single operator initially under supervision using the C60-Toshiba Core Vision with a 6-MHz endovaginal transducer. The thermal index for B-mode and Doppler examinations was <0.46. The velocity range, wall filter and colour gain were standardised for all scans. A 70 Hz filter was used to eliminate low frequency signals originating from vessel wall movements. Colour flow mapping and pulsed Doppler measurements were performed once normal pelvic findings were confirmed. Uterine artery, sub-endometrial and peri-follicular blood flow and vascularity were studied.

The uterus was visualised in a central longitudinal section. The maximum endometrial thickness between the echogenic interfaces of the myometrium and endometrium was measured. The endometrial pattern was graded in accordance with the method described by Gonen and Casper[382]: Type A, an entirely homogeneous, hyperechogenic pattern without a central echogenic line; Type B, an intermediate isoechogenic pattern with the same reflectivity as the surrounding myometrium and a non-prominent or absent central echogenic line; Type C, a multi-layered triple-line endometrium with a prominent outer and central hyperechogenic line and inner hypoechogenic regions.

Colour flow images of the ascending uterine artery were sampled bilaterally through the uterine isthmus in a coronal plane[261]. When satisfactory colour signals were obtained, blood flow velocity waveforms were recorded by placing the sample volume across the vessel and entering the pulsed Doppler mode. Areas of maximum colour intensity representing the greatest Doppler frequency shifts were selected for pulsed Doppler examinations. Only those samples avoiding wide angles of insonation ($>60^0$) were accepted for spectral analysis. The pulsatility index (PI= S-D/mean) was calculated automatically by the machine auto-trace facility, where S is the peak systolic Doppler shifted frequency, D is the minimum diastolic Doppler shifted frequency, and the mean is the mean maximum Doppler shifted frequency over the cardiac cycle. The PI has the advantage of reflecting blood flow impedance accurately, even in the absence of end diastolic blood flow[490]. Resistance index (RI= S-D/S), maximum

peak velocity (Vmax) and minimum diastolic velocity (Vmin) were also assessed.

Thereafter, sub-endometrial spiral arteries were identified by superimposing colour Doppler mapping over the 2-D image of the longitudinal section of the uterus at the level of maximum endometrial thickness. No correction was made for the angle of insonation of the Doppler beam but the presence of good colour signals was ensured within 1cm of endometrial-myometrial junction.

Colour flow mapping was used to localise perifollicular blood flow surrounding the selected follicle with the most extensive perifollicular vascular perfusion. The pulsed Doppler gate was placed over the vessel of interest and the recorded velocity waveforms were used for spectral analysis. No correction was made for the angle of insonation in the vessels but the highest achievable signals were sought over the maximum colour intensity. Subsequently, the vascularity of the follicle with the most extensive peri-follicular vascular perfusion was studied using power Doppler imaging unilaterally within the more responsive ovary. The percentage of the peri-follicular circumference that showed the contact vascularity with visible flow was recorded (Grade 1: <25%; Grade 2: 25-49%; Grade 3: 50-74%; Grade 4: 75-100%). Ovarian blood flow as either the ovarian artery flows towards the substance of the ovary[287] or within the ovarian stroma[259] was not analysed. Beacuse for such small and tortuous vessels, the angle of insonation cannot be determined and hence the reproducibility of Doppler indices was questioned[312]. Quantitative determination of oestradiol and FSH concentrations in serum was performed by immunoradiometric assay using the ADVIA® Centaur System™. Values were expressed in nmol/l and IU/l respectively. Intra- (2.0% to 2.9% for FSH; 2.2% to 5.5% for oestradiol) and inter- (1.2 to 2.7% for FSH; 2.7 to 5.2% for oestradiol) assay coefficients of variations were determined.

2.2.6 VEGF-VEGFR Concentrations

2.2.6.1 Blood Collection and Serum Preparation for VEGF-VEGFR

Blood samples were collected by venipuncture into 10 ml tubes and allowed to clot for 30 min before centrifugation at 1000 g for 15 min under temperature

control. To minimize the potential contribution of VEGF released from platelets during blood clotting centrifugation was not delayed[491]. Supernatant was divided into aliquots and stored at –80°C until measurement of VEGF and VEGFR concentrations.

2.2.6.2 Follicular Fluid Preparation for VEGF-VEGFR

From each woman, follicular fluid (FF) was collected from the first follicle punctured to avoid a dilution effect caused by follicle irrigation, which could artificially alter VEGF concentration. This follicle was selected as being the most regular and the largest. A wide interfollicular variation in intrafollicular steroid and cytokine concentrations is reported as a reflection of follicular asynchrony during ovarian stimulation for IVF[492,493]. Therefore, to eliminate the uncertainty originating from unquantifiable contribution from all individual follicles, it was elected to evaluate only the largest follicle separately. After identification and removal of the oocyte, the clear FF from each woman was centrifuged at 1000 g for 15 min to separate cellular contents and debris. When blood contamination was observed, follicular fluid was discarded. Follicular fluid supernatant was transferred to sterile polypropylene tubes and cryopreserved at –80°C for further analysis. All samples were assayed at the same time to avoid interassay variations.

2.2.6.3 Quantitative Determination of Free Human VEGF Concentrations in Serum and Follicular Fluid

The Quantikine VEGF Immunoassay (R & D System, Inc., Minneapolis, MN, USA) is a 4.5-hour solid phase enzyme-linked immunosorbent assay (ELISA) designed to measure VEGF165 levels. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of VEGF

bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

2.2.6.3.1 Assay Procedure

100 µL of assay-diluent and 100 µL of standard or sample were added to each well and incubated for two hours at room temperature. Each well was then aspirated and washed by filling with 400 µL of wash-buffer, repeating the process twice for a total of three washes. After the last wash, any remaining wash-buffer was removed by aspirating. The plate was inverted and blot dried against clean paper towels. 200 µL of VEGF-conjugate was added to each well and incubated for two hours at room temperature. Three cycles of aspiration and wash were performed. 200 µL of substrate-solution was added to each well and incubated for 25 minutes at room temperature by protecting from light. 50 µL of stop-solution was added to each well and to ensure thorough mixing the plate was gently tapped.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The average value of the duplicate readings for each standard and sample was calculated and the average zero standard optical density was subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. A best fit curve was drawn through the points on the graph. The minimum detectable dose was <9.0 pg/mL.

2.2.6.4 Quantitative Determination of Human Soluble VEGF Receptor Concentrations in Serum and Follicular Fluid

The Quantikine VEGF R1 immunoassay (R & D System, Inc., Minneapolis, MN, USA) is a 4.5hr solid-phase ELISA designed to measure human soluble VEGF R1. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF R1 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF R1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF R1 was added to the wells. Following a wash to remove any unbound

antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of VEGF R1 bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

2.2.6.4.1 Assay Procedure

100 µL of assay-diluent and 100 µL of standard or sample were added to each well and incubated for two hours at room temperature on a horizontal orbital microplate shaker set at 50 rpm. Each well was then aspirated and washed by filling with 400 µL of wash-buffer, repeating the process three times for a total of four washes. After the last wash, any remaining wash-buffer was removed by aspirating. The plate was inverted and blot dried against clean paper towels. 200 µL of VEGF R1-conjugate was added to each well and incubated for two hours at room temperature on the shaker. Four cycles of aspiration and wash were performed. 200 µL of substrate-solution was added to each well and incubated for 30 minutes at room temperature by protecting from light. 50 µL of stop-solution was added to each well and, to ensure thorough mixing, the plate was gently tapped. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The minimum detectable dose of VEGF R1 ranged from 1.63 - 14.4 pg/mL.

2.3 Frozen-Thawed Embryo Replacement (FTER) Treatment in Natural Cycle vs Hormonally Programmed Cycles

A prospective, randomised study with preference arm was conducted to evaluate if a natural cycle provides better hormonal milieu for endometrial receptivity than hormonally prepared cycles in ovulatory women undergoing FTER.

2.3.1 Patient Selection, Randomisation Process and Study Design

Women with clinical and laboratory evidence of ovulation (regular menstrual cycle between 26 to 35 days, mid-luteal progesterone levels ≥30 nmol/l) were invited to participate in the study. Couples who received oocyte or embryo donation or had logistic problems with daily visits for blood tests to either our Unit or their GP, were excluded from the study.

Randomisation was performed by drawing a sealed opaque envelope containing instructions on the treatment to be utilised. Treatments were randomly assigned to the envelopes by using a random number generator. Patients who were willing to participate in the study but had a strong preference for one particular type of treatment were allocated to the preference arm.

Demographic characteristics of the couples, clinical and embryological variables of the primary fresh IVF treatment cycles and subsequent FTER cycles were evaluated for their prognostic significance in the outcome of the index FTER cycles. For the variables found to be independent predictors of the treatment outcome of FTER in natural cycle (NC) and down-regulated replacement cycle (DRRC), the groups were compared to assure like-to-like evaluation of the two endometrial preparation methods. Clinical pregnancy was documented as the primary outcome measure. Serum VEGF-VEGFR concentrations and endometrial thickness and echo-pattern were also evaluated as prognostic determinants of the treatment outcome.

131 patients in each arm of the study would give an 80% chance of detecting a clinically significant difference in the clinical pregnancy rates from 15% to 30% at 5% level of statistical significance. With the anticipation of a 10% dropout rate the recruitment of 300 couples was planned.

2.3.2 IVF Protocol

Routine unit protocols for GnRH analogue down-regulated ovarian stimulation, oocyte collection, *in vitro* fertilization, cryopreservation and thawing routines were employed (refer to section 2.2.2 and *CD Rom 2.3.2*).

2.3.3 Natural Cycle (NC)

For 28-day menstrual cycles, cycle monitoring with blood tests for oestradiol (E_2) and LH concentration was commenced on cycle day 10. For longer menstrual cycles, this was commenced four days before the expected day of ovulation. If E_2 concentration was <0.2 nmol/l, the blood test was repeated after two days; if E_2 was between 0.2 and 0.5 nmol/l, the test was repeated after one day; and if E_2 was >0.5 nmol/l, tests were performed daily.

The LH surge was diagnosed when the LH concentration was at least twice that of the mean of the two preceding days with a concomitant drop in serum E_2 levels in the next sample. If no LH surge was detected after a reasonable length of time, progesterone (P_4) level was checked to evaluate if the surge was missed or the cycle was anovulatory. ET was scheduled on day 4 from the onset of LH surge. Patients outside the Grampian area and not able to attend the Unit for daily blood tests, posted their bloods to the Unit. Mid-luteal P_4 concentration was checked seven days after the ET.

2.3.4 Hormonally Programmed Cycle (DRRC)

Down-regulation with GnRH analogue was initiated on day 2 of the menstrual cycle and continued until day 15 of oestradiol valerate replacement when P_4 was added to the regimen. On day 18 of GnRH analogue treatment, serum oestradiol concentration was checked to confirm down-regulation. If oestradiol was <0.2 nmol/l HRT was started. Otherwise, GnRH analogue was continued for one more week before blood tests were repeated to confirm down-regulation.

Oestradiol valerate tablets were given as 2mg/day from day 1 (after confirmatory blood test of down-regulation) to day 10; 4mg/day from day 11 to day 14; and 6mg/day from day 15. Progesterone (Cyclogest suppositories) 400 mg twice daily (PV or per-rectal) was added from day 15. ET was performed on day 18 of the HRT. Serum beta hCG was measured on day 33 and, if positive, ultrasonography was performed after three weeks. If the pregnancy test was negative the hormone replacement was discontinued; if positive, the hormone replacement was continued until 12 weeks of pregnancy and gradually discontinued thereafter.

In both study groups, endometrial thickness and echo-pattern was assessed by vaginal ultrasonography and a blood sample was obtained for VEGF and VEGFR concentrations on the day of ET.

2.4 Statistics

Statistical analysis was performed by using SPSS software, version 10.0 (SPSS, Inc., Chicago, IL). Univariate analysis was performed to define the most probable predictors of pregnancy and multiple pregnancy. Multivariate analyses were conducted to define the best independent predictors of pregnancy and multiple pregnancy.

The κ^2 test was used to analyze nominal variables in the form of frequency tables. Fisher's exact test was computed when a table had a cell with an expected frequency < 5. Yates' corrected chi-square was computed for all other 2 by 2 tables. Normally distributed metric variables were tested with the t test. Ordinal variables or not-normally distributed metric variables were analyzed with the Mann-Whitney U test. If more than two groups had to be analyzed, normally distributed metric variables with equal variances (Levene test) were examined by means of one-way ANOVA test. Multiple comparisons were made with Bonferroni test. For not-normally distributed metric variables or for variables with unequal variances, the Kruskal-Wallis one-way ANOVA test for ranks was employed. For correlation analysis, Spearman's rank correlation coefficient was used. All tests were two-tailed with a confidence level of 95% ($p<0.05$). Logistic regression analyses were done by using forward stepwise and forced entry techniques. Regression coefficient (β_n), regression constant (β_0) and odds ratio (OR) was documented. Cumulative probability of the standardized residuals was plotted to assess the normal distribution. Goodness of fit of the model was assessed by R^2 statistics.

Regression constant is the $\log_e$ transformation of the odds of an event occurring when all independent predictors are zero. The odds of an event can be defined as the ratio of the probability of an event occurring to the probability of an event not occurring. The odds ratio is the change in the odds for an event when the value of the independent variable increases by 1 unit. The logistic regression equation for an event occurring can be written as 'Probability (event)=1 / 1 + $e^{-Z'}$, where e is the base of the natural logarithms, 2.718 and Z is the linear combination of '"$Z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n$'.and X_n is the independent variable.

The predictive value of prognostically significant parameters was calculated by receiver operating characteristics (ROC) curve analysis. Receiver-operating characteristic curves represent the probability of true positive results (sensitivity) as a function of the probability of false positive results (1 - specificity). The decision criterion (cut-off point) was set at each different point of the decision axis and sensitivity and specificity for these points were calculated. When the cut-off points were varied, the possible combinations of sensitivity and specificity obtained were combined to calculate the area under the curve (AUC). The AUC, calculated as [sensitivity / (1 - specificity)], is a measure that shows how 'good' (AUC close to 1) or 'bad' (AUC close to 0.5) a test is.

3 Factors Affecting IVF Outcome: Results

3.1 Descriptive Statistics of Patient Characteristics, Clinical Variables of IVF (Ovarian Stimulation) and Treatment Outcome

Demographic characteristics of the couples, diagnostic characteristics of their subfertility, response parameters to the ovarian stimulation and treatment outcome were summarized (Table 3.1, 3.2, 3.3). The analysis is based on 12,332 IVF treatment cycles; however, the whole data were not available for each variable. Couples may have more than one aetiology of subfertility, hence the cumulative percentage was >100% (Table 3.2).

Table 3.1: Patient Characteristics and Clinical Variables of IVF

Clinical Variables		Mean ± SD	N
Female Age (year)		35.2 ± 4.9	10827
Duration of Subfertility (year)		5.3 ± 3.6	8354
Previous Pregnancy		0.6 ± 1.1	10827
Previous IVF Cycles		1.8 ± 1.4	10827
Early Follicular Phase FSH (IU/l)		6.6 ± 3.6	4336
Early Follicular Phase E_2 (nmol/l)		0.24 ± 0.52	2544
Total Gonadotrophin Dose (IU)		2851 ± 1351	8427
Duration of Ovarian Stimulation (day)		11.0 ± 3.1	8425
Number of Follicles Measuring	8-12 mm	3.4 ± 3.0	8488
	13-16 mm	4.1 ± 3.1	8488
	17-19 mm	3.7 ± 2.6	8488
	≥ 20 mm	1.8 ± 2.2	5928
hCG Day E_2 (nmol/l)		5.92 ± 4.27	4206
Number of Oocytes Collected		8.7 ± 5.6	10752
Number of 2-PN Embryos		5.1 ± 4.0	10752
Number of Embryos Transferred		2.3 ± 1.0	10752
Number of Embryos Implanted		0.4 ± 0.7	9701
Endometrial Thickness (mm)		11.0 ± 1.8	8363

N: Number of subjects whose data were available for each variable, SD: Standard Deviation, PN: Pro-nuclear

Table 3.2: Aetiology of Subfertility

	N	Percent
Male Factor	4799	44.3
Unexplained	3125	28.9
Tubal Factor	3229	29.8
Endometriosis	1128	10.4
PCOS	878	8.1
Cervical Factor	78	0.7

The frequency distribution of female age, duration of subfertility, gravidity, pre-stimulation FSH concentrations, number of previous IVF attempts, cyst development after down-regulation, detection of hydrosalpinx on ultrasonography (USG), endometrial thickness on the last day of ovarian stimulation, and number of embryos transferred and implanted are given on the CD Rom *(Table 3.1-3.10CD).*

Table 3.3: Treatment Outcome

	N	Percent
No-Embryo Transfer	983	9.2
Ongoing Pregnancy	2477	23.2
No Pregnancy	6456	60.4
Miscarriage	559	5.2
Ectopic Pregnancy	69	0.6
Biochemical Pregnancy	150	1.4
Total	10694	100.0

3.1.1 Univariate Analysis

The following variables differed significantly between women with ongoing pregnancy and no-ongoing pregnancy: Female age, duration of subfertility, early follicular FSH, previous IVF attempts, total gonadotrophin dose, average daily gonadotrophin dose, number of developing follicles (except ≥20mm), number of oocytes collected, number of 2-PN embryos created, number of embryos transferred, number of embryos cryopreserved, and endometrial thickness (Table 3.4).

In the presence of an ovarian cyst following down-regulation, the probability of no-ET (p: <0.01) and no-ongoing pregnancy (p: 0.09) was higher but only the former had statistical significance. A sonographically visible hydrosalpinx decreased the probability of ongoing pregnancy (p: 0.05) without affecting the probability of no-ET (p: 0. 12) (*Table 3.11, 3.12, 3.14, 3.15CD*).

Table 3.4: Patient Characteristics and Clinical Variables of IVF for Ongoing Pregnancy

		Ongoing Pregnancy	N	Mean ± SD	p
Female Age (year)		No	7224	35.6 ± 4.8	<0.01
		Yes	2474	33.6 ± 4.0	
Duration of Subfertility (year)		No	5627	5.4 ± 3.7	<0.01
		Yes	1980	4.7 ± 3.1	
Previous Pregnancy		No	7235	0.6 ± 1.1	NS
		Yes	2476	0.6 ± 1.0	
Total Gonadotrophin Dose (IU)		No	5555	2911 ± 1257	<0.01
		Yes	2042	2516 ± 1090	
Number of Oocytes Collected		No	7181	9.8 ± 5.8	<0.01
		Yes	2476	11.5 ± 5.7	
Previous IVF Cycles		No	7235	1.9 ± 1.4	<0.01
		Yes	2476	1.7 ± 1.1	
Number of Follicles Measuring	8-12 mm	No	5580	3.3 ± 3.0	<0.01
		Yes	2054	3.8 ± 3.1	
	13-16 mm	No	5580	4.0 ± 2.9	<0.01
		Yes	2054	4.9 ± 3.2	
	17-19 mm	No	5580	3.7 ± 2.6	<0.01
		Yes	2054	4.3 ± 2.7	
	≥ 20 mm	No	3834	1.8 ± 2.2	NS
		Yes	1478	1.9 ± 2.0	
Endometrial Thickness (mm)		No	5495	11.0 ± 1.7	<0.01
		Yes	2031	11.4 ± 1.7	
hCG Day E_2 (nmol/l)		No	2711	6.07 ± 4.52	NS
		Yes	1082	6.13 ± 3.40	
Pre stimulation FSH (IU/l)		No	2850	6.6 ± 3.3	<0.01
		Yes	1038	6.2 ± 2.8	
Number of 2-PN Embryos		No	7181	5.2 ± 3.7	<0.01
		Yes	2476	6.7 ± 3.8	
Number of Embryos Transferred		No	7181	2.5 ± 0.7	<0.01
		Yes	2476	2.6 ± 0.5	
Number of Embryos Cryopreserved		No	7181	1.0 ± 2.6	<0.01
		Yes	2476	1.9 ± 3.3	

NS: Not significant, p: Significance value

3.1.2 Multivariate Analysis

Using the forward conditional stepwise entry method, logistic regression analysis was performed with the following independent variables to predict ongoing pregnancy in 2109 cycles: Female age, gravidity, early follicular phase FSH concentration, aetiology of subfertility, duration of subfertility, number of previous IVF attempts, ultrasonographic appearance of PCO and of hydrosalpinx, type of luteal support, total gonadotrophin dose required for ovarian stimulation, average gonadotrophin dose per day, duration of ovarian stimulation, duration of coasting, E_2 level before hCG administration, sperm concentration, number of oocytes collected, number of oocytes used for fertilisation, number of 2-PN embryos created, number of embryos transferred, use of assisted hatching, and number of embryos cryopreserved.

Female age, duration of subfertility, total gonadotrophin dose, number of oocytes collected, number of 2-PN embryos created, and number of embryos transferred were found to be significant (Table 3.5).

Table 3.5: Patient Characteristics and Clinical Variables of IVF in the Equation for Ongoing Pregnancy

	Regression Coefficient	SE	OR	p
Age	-0.11	0.01	0.90	<0.01
Duration of Subfertility	-0.04	0.02	0.96	<0.05
Total Gonadotrophin Dose	-0.03	0.01	0.97	<0.01
Oocytes Collected	0.07	0.02	0.93	<0.01
Number of 2-PN Embryos	0.12	0.02	1.13	<0.01
Number of ET	0.27	0.09	1.31	<0.01
Constant	3.20	0.49	24.42	<0.01

SE: Standard error, ET: Number of embryos transferred, OR: Odds ratio

The same analyses were repeated for the ongoing pregnancy, miscarriage, biochemical pregnancy, no-ET, and no-pregnancy outcomes *(Table 3.13, 3.16-3.18CD).*

3.2 Descriptive Statistics of Variables of IVF (Oocytes)

Number and percentages of the oocytes collected at varying levels of maturity were summarized (Table 3.6).

Table 3.6: Descriptive Statistics of Oocytes

		Mean ± SD	N
Number of	Aspirated Follicles	11.7 ± 6.4	8561
	Collected Oocytes	9.8 ± 5.9	12332
	Mature Oocytes	4.9 ± 4.1	12068
	Borderline Mature Oocytes	3.5 ± 2.8	12068
	Immature Oocytes	0.9 ± 1.4	12068
	Post-mature Oocytes	0.2 ± 0.9	9454
% of	Collected Oocytes	0.8 ± 0.1	8567
	Mature Oocytes	0.4 ± 0.2	12046
	Borderline Mature Oocyte	0.3 ± 0.2	12046
	Immature Oocytes	0.1 ± 0.1	12046
	Post-mature Oocytes	0.02 ± 0.09	9432

% of collected oocytes : 'number of oocytes collected:number of follicles aspirated' ratio
% of mature oocytes : 'number of mature oocytes number of oocytes collected' ratio
Percentages represented as decimal of 1.0 ± SD

3.2.1 Factors Related to Oocyte Maturity

Patients' characteristics and clinical variables of ovarian stimulation, which correlated significantly with the number of mature, immature and post-mature oocytes retrieved were summarized (Table 3.7). Positive correlation between the variables was given as "↑", negative correlation as "↓", and no correlation as "↔". Numerical values of the correlation coefficients and corresponding p values were detailed on the CD Rom *(Table 3.19, 3.20CD).*

For the number of mature oocytes collected, the correlation coefficient of follicles measuring 13-19mm was twice that of follicles measuring <13mm and six times that of follicles measuring >20mm. For the number of immature oocytes collected, the highest correlation coefficient was with follicles measuring 8-16mm.

Table 3.7: Correlations between Patient Characteristics, Clinical Variables of IVF and the Number and % of Oocytes Collected

		Number of Oocytes		
		Mature	**Immature**	**Post-mature**
Female Age		↓	↓	↑
Duration of Subfertility		↔	↓	↔
Previous Pregnancy		↑	↓	↑
Total Gonadotrophin Dose		↓	↓	↑
Duration of Ovarian Stimulation		↓	↑	↔
Follicles	8-12mm	↑	↑	↑
	13-16mm	↑	↑	↑
	17-19mm	↑	↑	↔
	≥ 20mm	↑	↑	↑
hCG Day E_2		↑	↑	↑
Pre-stimulation FSH		↓	↓	↑
E_2 per Follicle		↔	↔	↑
Number of Oocytes Collected		↑	↑	↑
Number of 2-PN Embryos		↑	↑	↑
Number of Embryos Cryopreserved		↑	↑	↑
Number of Embryos Implanted		↑	↔	↔
				% of Oocytes
		Mature	**Immature**	**Post-mature**
Female Age		↓	↓	↑
Number of Oocytes Collected		↑	↑	↑
Duration of Subfertility		↔	↓	↔
Total Gonadotrophin Dose		↓	↑	↑
Duration of Ovarian Stimulation		↔	↑	↔
E_2 Level on the day of hCG		↔	↑	↑
Pre-stimulation FSH		↓	↔	↑
Number of 2-PN Embryos		↑	↑	↑
Number of Embryos Implanted		↑	↓	↔

↑ : Positive correlation between the variables, ↓ : Negative correlation, ↔ : No significant correlation.

The following variables differed significantly when women with PCOS and women without PCOS were respectively compared; % of oocytes collected (86% vs 88%), % of borderline (34% vs 37%), immature (11% vs 9%), post-mature (4% vs 2%) oocytes, number of oocytes collected (11.5 vs 10), number of follicles aspirated (13.2 vs 11.5), number of mature (5.7 vs 5), borderline (3.8 vs 3.5), immature (1.3 vs 0.9), and post-mature (0.5 vs 0.2) oocytes collected *(Table 3.21CD).*

In women with and without endometriosis, the respective percentage of oocytes collected (86% vs 88%) and the percentage (4% vs 2%) and number (0.3 vs 0.2) of post-mature oocytes collected, differed significantly. Among Grades 1, 2, 3 and 4 endometriosis, the multiple comparison tests revealed that the following variables differed significantly between Grades 1 and 3 endometriosis respectively; % of oocyte collected (88% vs 83%), number of follicles aspirated (11.7 vs 10.2), number of oocytes collected (10.5 vs 8.8), and number of borderline oocytes collected (3.7 vs 3) *(Table 3.22, 3.23CD).*

The following variables differed significantly between women with unexplained subfertility and women with a specific subfertility diagnosis, respectively: % of oocytes collected (87% vs 88%), % of mature (48% vs 50%) and post-mature (4% vs 2%) oocytes collected, number of oocytes collected (9.5 vs 10.1), number of follicles aspirated (11 vs 12), and number of mature (4.8 vs 5.2), borderline (3.4 vs 3.6), and post-mature (0.3 vs 0.2) oocytes collected *(Table 3.24CD).*

The following variables differed significantly between women with and without ovarian cysts following down-regulation, respectively: % of oocytes collected (85% vs 88%), number of oocytes collected (9.7 vs 10.4), and number of post-mature oocytes collected (0.1 vs 0.3) *(Table 3.25CD).* The number of follicles aspirated (10.7 vs 12) insignificantly differed between women with and without hydrosalpinx on USG *(Table 3.26CD).*

3.2.2 Univariate Analysis

The following variables differed significantly when women with ongoing pregnancy and no-ongoing pregnancy were compared: % of oocyte collected, % of mature, borderline, immature oocytes collected, number of follicles aspirated, number of oocytes collected, and number of mature and borderline oocytes collected (Table 3.8).

Table 3.8: Oocyte Maturity for Ongoing Pregnancy

	Ongoing Pregnancy	N	Mean ± SD	p
% of Oocytes from Follicular Aspirate	No	5628	0.8 ± 0.1	<0.01
	Yes	2068	0.9 ± 0.1	
% of Mature Oocytes	No	7003	0.4 ± 0.2	<0.01
	Yes	2437	0.5 ± 0.2	
% of Borderline Oocyte	No	7003	0.37 ± 0.2	<0.01
	Yes	2437	0.36 ± 0.2	
% of Immature oocytes	No	7003	0.1 ± 0.1	<0.01
	Yes	2437	0.09 ± 0.12	
Number of Aspirated Follicles	No	5626	11.5 ± 6.2	<0.01
	Yes	2068	13.1 ± 6.0	
Number of Oocytes Collected	No	7182	9.8 ± 5.8	<0.01
	Yes	2476	11.5 ± 5.7	
Number of Mature Oocytes	No	7005	4.9 ± 4.1	<0.01
	Yes	2437	6.1 ± 4.3	
Number of Borderline Mature Oocytes	No	7005	3.5 ± 2.8	<0.01
	Yes	2437	4.0 ± 3.0	
% of Post-mature Oocytes	No	5201	0.02 ± 0.09	NS
	Yes	1810	0.02 ± 0.08	
Number of Immature Oocytes	No	7005	0.9 ± 1.4	NS
	Yes	2437	1.0 ± 1.5	
Number of Post-mature Oocytes	No	5203	0.2 ± 1.0	NS
	Yes	1810	0.2 ± 1.1	

Percentages represented as decimal of 1.0 ± SD

The same analysis was performed for the pregnancy, miscarriage, biochemical pregnancy, no-ET, and no-pregnancy outcomes. Percentage of mature, borderline mature, immature, post-mature oocytes, % of oocytes collected from follicular aspirate, number of aspirated follicles, number of oocytes collected, and number of mature, borderline mature, and immature oocytes differed significantly among these different outcomes groups *(Table 3.27CD).*

3.2.3 Multivariate Analysis

In 7013 cycles, logistic regression analysis was performed using the forward stepwise conditional entry method to evaluate the prognostic significance of the number of mature, borderline, immature, and post-mature oocytes in the prediction of ongoing pregnancy. Prognostic significance was found in the number of mature and borderline oocytes (Table 3.9).

Table 3.9: Oocyte Maturity in the Equation for Ongoing Pregnancy

	Regression Coefficient	SE	OR	p
Mature oocytes	0.06	0.01	1.06	<0.01
Borderline Mature Oocyte	0.05	0.01	1.05	<0.01
Constant	-1.56	0.05	0.21	<0.01

3.3 Descriptive Statistics of Variables of IVF (Fertilisation, Individual Embryo Quality)

Statistical details of the fertilisation related variables and the morphological development of the individual embryos graded 1 to 6 (G) and developmental stages 1 to 9 (S) (indicating the number of blastomeres) were summarized (Table 3.10, 3.11).

Table 3.10: Fertilisation Variables

	Mean ± SD	N
Number of Oocytes	9.4 ± 5.5	8090
Number of Fertilised Oocytes (2-PN)	6.2 ± 4.2	8090
Number of Cleavage Stage Embryos	4.9 ± 3.2	8090
% of Fertilisation	0.6 ± 0.2	8090
% of Cleavage	0.8 ± 0.2	8090

% of Fertilisation: ratio of oocytes fertilized to oocytes used % of Cleavage: ratio of embryos cleaved to 2-PN embryos

Table 3.11: Number of Embryos at Various Levels of Development

Embryo Stage and Grade	Mean ± SD	N
S2G1	0.3 ± 0.8	8090
S2G2	0.6 ± 1.1	8090
S2G3	0.2 ± 0.7	8090
S2G4	0.1 ± 0.4	8090
S2G5	0.02 ± 0.21	8090
S3G1	0.09 ± 0.35	8090
S3G2	0.2 ± 0.5	8090
S3G3	0.1 ± 0.5	8090
S3G4	0.07 ± 0.34	8090
S3G5	0.01 ± 0.11	8090
S4G1	0.5 ± 1.1	8090
S4G2	0.8 ± 1.3	8090
S4G3	0.3 ± 0.8	8090
S4G4	0.1 ± 0.5	8090
S4G5	0.03 ± 0.33	8090
S6G1	0.1 ± 0.5	8090
S6G2	0.4 ± 0.9	8090

Embryo Stage and Grade	Mean ± SD	N
S6G3	0.2 ± 0.6	8090
S6G4	0.04 ± 0.30	8090
S6G5	0.01 ± 0.16	8090
S8G1	0.09 ± 0.45	8090
S8G2	0.1 ± 0.6	8090
S8G3	0.03 ± 0.24	8090
S8G4	0.01 ± 0.12	8090
S8G5	0.0 ± 0.0	8090
S9G1	0.0 ± 0.0	8090
S9G2	0.01 ± 0.09	8090
S9G3	0.0 ± 0.0	8090
S9G4	0.0 ± 0.0	8090
S9G5	0.0 ± 0.0	8090

S: Stage of the embryo, G: Grade of the embryo. Grade 6 refers to non-cleaved embryos, and hence not included .

3.3.1 Univariate Analysis

There were significant differences when embryological variables were evaluated for their distribution between cycles with and without ongoing pregnancy: number of oocytes used for fertilisation, number of oocytes fertilised, and number of cleavage stage embryos. Although fertilisation rates differed between the ongoing pregnancy and no-ongoing pregnancy groups, cleavage rates were similar in both groups (Table 3.12). At almost all stages of embryo development (S_2 to S_8), there were more Grade 1 and 2 embryos in the ongoing pregnancy group (*Table 3.28CD*). The number of Grade 3 embryos had positive prognostic significance but mostly if cellular division was at S_2 to S_4. The number of Grade 4 and 5 embryos was almost always higher in the non-pregnant group but not at a level of significance.

Table 3.12: Fertilisation Variables for Ongoing Pregnancy

	Ongoing Pregnancy	N	Mean ± SD	p
Number of Oocytes	No	5578	9.0 ± 5.4	<0.01
	Yes	2054	10.7 ± 5.3	
Number of Fertilized Oocytes (2-PN)	No	5578	5.9 ± 4.1	<0.01
	Yes	2054	7.5 ± 4.1	
Number of Cleavage Stage Embryos	No	5578	4.7 ± 3.1	<0.01
	Yes	2054	6.1 ± 3.0	
% of Fertilization	No	5578	0.67 ± 0.2	<0.01
	Yes	2054	0.72 ± 0.2	
% of Cleavage	No	5578	0.85 ± 0.2	NS
	Yes	2054	0.85 ± 0.1	

3.3.2 Multivariate Analysis

A logistic regression analysis using the forward stepwise conditional entry method was performed on 3616 cycles to predict ongoing pregnancy with the following variables as independent predictors: number of oocytes used for fertilisation, number of oocytes fertilised, number of cleavage stage embryos, fertilisation rate, cleavage rate, and number of embryos at different developmental stages (S) and grades (G) (S_2G_1 to S_9G_5).

The number of oocytes used for fertilisation, fertilisation rate and embryo cleavage rate positively affected the outcome. The number of slow dividing embryos with either good or poor grading, and the number of fast dividing embryos with poor grading, both had a significant negative effect on the outcome. The number of four-cell stage embryos with good morphological grading had the largest positive effect on the probability of ongoing pregnancy (Table 3.13).

Table 3.13: Fertilisation Variables in the Equation for Ongoing Pregnancy

	Regression Coefficient	SE	OR	p
Number of Oocytes	0.06	0.01	1.07	<0.01
% of Fertilization	1.27	0.19	3.54	<0.01
% of Cleavage	0.91	0.21	2.49	<0.01
S2G1	-0.11	0.04	0.90	0.01
S3G4	-0.28	0.12	0.76	<0.05
S4G1	0.08	0.03	1.09	<0.05
S4G2	0.06	0.03	1.06	<0.05
S4G4	-0.43	0.12	0.65	<0.01
S8G3	-0.56	0.28	0.57	<0.05
Constant	-3.25	0.28	0.04	<0.01

3.4 Univariate Analysis of the Global Quality of the Whole Embryo Cohort

Total number and % of good (Grades 1 and 2) and poor (Grades 3, 4 and 5) grade embryos created in the whole embryo cohort differed significantly between women with ongoing pregnancy and with no-ongoing pregnancy (Table 3.14). Number of good grade embryos appeared to have more clinical significance due to larger differences between pregnant and non-pregnant women.

Table 3.14: Good-Poor Grade Embryos for Ongoing Pregnancy

		Ongoing Pregnancy	N	Mean ± SD	p
Number of	Good Grade Embryos	No	5577	3.1 ± 2.7	<0.01
		Yes	2054	4.3 ± 2.8	
	Poor Grade Embryos	No	5578	1.5 ± 2.0	0.01
		Yes	2054	1.7 ± 2.1	
% of	Good Grade Embryos	No	5541	0.6 ± 0.3	<0.01
		Yes	2050	0.7 ± 0.2	
	Poor Grade Embryos	No	5542	0.3 ± 0.3	<0.01
		Yes	2050	0.2 ± 0.2	

To assess the predictive power of the total number of good grade embryos for ongoing pregnancy, an ROC curve analysis under non-parametric assumptions was performed on 2054 cycles with ongoing pregnancy and 5577 cycles with no ongoing pregnancy. The area under the curve 0.64 signified a prognostic significance beyond a chance factor (p:>0.01). Sensitivity and specificity values were detailed in the table with corresponding numbers of good grade embryos *(Table 3.52CD).*

An arbitrary cut-off point of three good grade embryos was chosen to sub-categorise the study population. In the group with >3 good grade embryos the probability of ongoing pregnancy was significantly higher than in the group with ≤3 good embryos (36% vs 20%) *(Table 3.53CD).* Test sensitivity for ongoing pregnancy was 55-72%.

3.5 Descriptive Statistics of Variables of IVF (Quality of the Transferred Embryos and Embryo Transfer Procedure)

Descriptive details of the morphological development of the transferred embryos graded 1 to 6 and developmental stages 1 to 9 (indicating the number of blastomeres) were given (Table 3.15). Descriptive details of the tip of the transfer catheter, tenaculum and stylet use, degree of discomfort and procedural difficulty, and USG guidance during ET were detailed in the CD Rom *(Table 3.29-3.34CD).*

Table 3.15: Clinical and Embryological Variables of Embryo Transfer

	Mean ± SD	N
Grade of the First Transferred Embryo	1.7 ± 0.7	8013
Stage of the First Transferred Embryo	4.8 ± 1.7	8003
Grade of the Second Transferred Embryo	1.9 ± 0.8	7261
Stage of the Second Transferred Embryo	4.4 ± 1.6	7254
Grade of the Third Transferred Embryo	2.0 ± 0.8	5032
Stage of the Third Transferred Embryo	4.0 ± 1.6	5032
Number of Embryos Transferred	2.5 ± 0.6	9931
Embryo Transfer Time (sec)	53.6 ± 11.8	7345

3.5.1 Factors Related to the Quality of Transferred Embryos

Patients' characteristics and clinical variables of IVF, which correlated significantly with the grade and the stage of the transferred embryos were summarized (Table 3.16). Positive correlation between the variables was given as "↑", negative correlation as "↓", and no correlation as "↔". Numerical values of the correlation coefficients and corresponding p values were detailed in the CD Rom *(Table 3.35-3.37CD).*

Table 3.16: Correlations between Patient Characteristics, Clinical Variables of IVF and the Quality of Embryos Transferred

	1st Embryo		2nd Embryo	
	Grade	Stage	Grade	Stage
Female age	↑	↔	↔	↔
Total gonadotrophin dose	↑	↔	↑	↓
Duration of ovarian stimulation	↔	↓	↔	↓
E_2 level on the day of hCG administration	↓	↑	↓	↑
Number of oocytes collected	↓	↑	↓	↑
Number of 2-PN embryos created	↓	↑	↓	↑
Number of cleavage stage embryos	↓	↑	↓	↑
Number of embryos cryopreserved	↓	↑	↔	↑
Number of embryos implanted	↓	↑	↓	↑
Stage of the 1st Embryo transferred	↑		↔	↑
Stage of the 2nd Embryo transferred	↓	↑	↔	
Stage of the 3rd Embryo transferred	↓	↑	↓	↑
Grade of the 1st Embryo transferred		↔	↑	↓
Grade of the 2nd Embryo transferred	↑	↔		↔
Grade of the 3rd Embryo transferred	↑	↔	↑	↓

↑ : Positive correlation between the variables, ↓ : Negative correlation, ↔ : No significant correlation.

3.5.2 Univariate Analysis

When women with ongoing pregnancy and no-ongoing pregnancy were compared, there were significant differences in the following variables respectively: ET time (52 sec vs 54), number of embryos transferred (2.6 vs 2.5), stage of the first (5 vs 4.8), second (4.6 vs 4.3) and third (4.2 vs 3.9) embryos transferred, and grade of the first (1.6 vs 1.8), second (1.8 vs 2), and third (1.9 vs 2.1) embryos transferred *(Table 3.38CD).*

The ongoing pregnancy rate was not affected by the condition of the catheter tip after the transfer, degree of discomfort experienced by the woman during the transfer, degree of technical difficulty of the transfer as assessed by the physician, or the use of tenaculum, stylet or ultrasound guidance (*Table 3.39-3.43CD).* There was no link between the duration of the ET and its degree of technical difficulty *(Table 3.44CD).*

Biochemical pregnancy, miscarriage, ectopic pregnancy and ongoing pregnancy outcomes could not be differentiated by grade and stage of the best three embryos transferred or the total number of good (1 and 2) and poor (3, 4 and 5) grade embryos in the cohort *(Table 3.45CD).*

3.5.3 Multivariate Analysis

Logistic regression analysis using the forward selective conditional entry method was performed on 4953 cycles, to evaluate the individual contribution in the prediction of ongoing pregnancy rates, of the number, grade and stage of the embryos transferred.

The stage and grade of the first, grade of the second, and stage of the third embryos transferred were significant predictors of ongoing pregnancy (Table 3.17, 3.18). After correction for embryo quality, the number of embryos transferred was no longer a significant determinant of the treatment outcome (p: 0.69).

Table 3.17: Embryo Quality in the Equation for Ongoing Pregnancy

	Regression Coefficient	SE	OR	p
Grade of the 1st Embryo	-0.12	0.05	0.88	<0.05
Stage of the 1st Embryo	0.08	0.03	0.92	<0.01
Grade of the 2nd Embryo	-0.16	0.05	0.85	<0.01
Stage of the 3rd Embryo	0.16	0.03	1.17	<0.01
Constant	-0.65	0.13	0.52	<0.01

Table 3.18: Variables not in the Equation for Embryo Quality and Ongoing Pregnancy

	p
Number of Embryos Transferred	NS
Stage of the 2nd Embryo	NS
Grade of the 3rd Embryo	NS

3.6 Multivariate Analysis of Patient Characteristics, Clinical Variables of IVF: Ovarian Stimulation, Oocyte Quality, Fertilisation, Individual Embryo Quality, Transferred Embryo Quality for Ongoing Pregnancy

Logistic regression analysis using the forward stepwise conditional entry method and the number of embryos transferred as the constant, was performed on 515 cycles to assess the prognostic significance of different clinical and embryological variables in the prediction of ongoing pregnancy (Table 3.19, 3.20).

Table 3.19: Independent Variables used in Logistic Regression Analysis

Female Age	S2G1
Duration of Subfertility	S2G2
Number of Previous IVF Attempts	S3G1
Pre Stimulation FSH	S3G2
Total Dose of Gonadotrophin	S4G1
Duration of Ovarian Stimulation	S4G2
hCG day E_2	S6G1
Number of Aspirated Follicles	S6G2
Number of Collected Oocytes	Grade and Stage of the 1st Embryo
Number of Mature Oocytes	Grade and Stage of the 2nd Embryo
Number of Borderline Oocytes	Grade and Stage of the 3rd Embryo
Number of 2PN Embryos	Number of Cleavage Stage Embryos
	Number of Cryopreserved Embryos

Table 3.20: Variables in the Equation for Ongoing Pregnancy

	Regression Coefficient	SE	OR	p
Female Age	-0.13	0.03	0.88	<0.01
Number of Aspirated Follicles	0.06	0.02	0.94	0.01
Grade of the 2nd Embryo	-0.32	0.15	0.73	<0.05
Stage of the 3rd Embryo	0.18	0.08	1.20	<0.05
S2G2	0.27	0.08	1.31	<0.01
S4G2	0.22	0.08	1.25	<0.01
Constant	3.74	1.07	41.87	<0.01

Inclusion of the % expression of the maturity of oocytes collected and fertilised did not alter the most prognostic variables found in the first analysis *(Table 3.46-3.48CD).*

3.7 Prognostic Significance of Number of Embryos Transferred for Ongoing Pregnancy with Consideration of Quality of the Individual Embryos and the Whole Embryo Cohort (Number of Good Grade Embryos)

Logistic regression analysis was performed to predict ongoing pregnancy rate by using female age, number of embryos transferred, and number of embryos at different grade and stage of cell division as independent variables in couples who had ≤3 cleavage stage embryos available for transfer and in couples who had >3 cleavage stage embryos. The forward stepwise conditional entry method was employed.

In the group with ≤3 cleavage stage embryos available for ET, number of embryos transferred was not found to be prognostic once the effect of female age and embryo quality in terms of number of embryos at different stage of development and morphological grade were controlled. In the group with >3 cleavage stage embryos available for ET, the number of embryos transferred had a negative influence on the ongoing pregnancy rates where higher numbers were associated with lower pregnancy rates (Table 3.21).

Table 3.21: Cleavage Stage Embryos in the Equation for Ongoing Pregnancy

Cleavage Stage Embryos		Regression Coefficient	SE	OR	p
≤3	Age	-0.11	0.02	0.90	<0.01
	S4G1	0.59	0.16	1.80	<0.01
	S4G2	0.48	0.12	1.61	<0.01
	S2G2	0.37	0.13	1.45	0.01
	S3G1	0.72	0.37	2.05	0.05
	S4G4	-1.80	1.02	0.17	NS
	S9G2	1.29	0.68	3.62	NS
	Constant	1.61	0.63	4.99	0.01
>3	Age	-0.09	0.01	0.92	<0.01
	Number of ET	-0.28	0.10	0.76	<0.01
	S2G1	-0.12	0.05	0.89	0.01
	S3G4	-0.37	0.13	0.69	<0.01
	S4G4	-0.43	0.12	0.65	<0.01
	S8G3	-0.59	0.30	0.56	0.05
	Constant	3.31	0.38	27.32	<0.01

Total number of good grade (1 and 2) embryos created in the whole embryo cohort and the number of embryos transferred differed significantly between women with ongoing pregnancy and with no-ongoing pregnancy (Table 3.22).

Table 3.22: Number of Embryos Transferred, Good-Poor Grade Embryos for Ongoing Pregnancy

	Ongoing Pregnancy	N	Mean ± SD	p
Number of Embryos Transferred	No	7181	2.5 ± 0.7	<0.01
	Yes	2476	2.6 ± 0.5	
Good Grade Embryos Grade 1, 2	No	5577	3.1 ± 2.7	<0.01
	Yes	2054	4.3 ± 2.8	

Logistic regression analysis was performed to predict the ongoing pregnancy rate by using female age and number of embryos transferred as independent variables in couples who had ≤3 good grade embryos available for transfer and in couples who had >3 good grade embryos. The forward stepwise conditional entry method was employed.

In the group with ≤3 good grade embryos available for ET, the number of embryos transferred was found to be prognostic. In the group with >3 good grade embryos available for ET, the number of embryos transferred had a

negative influence on the ongoing pregnancy rates where higher numbers were associated with lower pregnancy rates (Table 3.23).

Table 3.23: Number of Embryos Transferred, Good-Poor Grade Embryos in the Equation for Ongoing Pregnancy

Good Grade Embryos		Regression Coefficient	SE	OR	p
≤3	Age	-0.09	0.00	0.91	<0.01
	Number of Embryos Transferred	0.42	0.05	1.52	<0.01
	Constant	0.87	0.31	2.40	<0.01
>3	Age	-0.07	0.00	0.92	<0.01
	Number of Embryos Transferred	-0.24	0.08	0.78	<0.01
	Constant	2.68	0.33	14.67	<0.01

3.7.1 Analysis of Variables of IVF (Number of Cleavage Stage Embryos, Good Grade Embryos and Transferred Embryos) for Ongoing Pregnancy

If there were ≤3 cleavage stage embryos available for transfer but none of good grade, a higher number of embryos transferred was associated with higher probability of ongoing pregnancy. If at least one embryo was of good grade there was no benefit in increasing the number of embryos transferred. This was more evident if all three embryos were of good grade. If there were >3 cleavage stage embryos available for transfer but none of good grade, then transferring a higher number of embryos was associated with an insignificantly higher probability of pregnancy. If at least one of the embryos was of good grade there was clearly no benefit in increasing the number of embryos transferred. This was more evident if three or more embryos were of good grade (Table 3.24).

Table 3.24: Cleavage Stage and Good Grade Embryos for the Number of Embryos Transferred (Continuous Variable)

Number of			Number of Embryos Transferred			
Cleavage Stage Embryos	**Good Grade Embryos**	**Ongoing Pregnancy**	**N**	**Mean**	**SD**	**p**
≤3	0	No	537	1.8	0.8	0.01
		Yes	36	2.2	0.7	
	1	No	811	1.6	0.7	NS
		Yes	119	1.7	0.7	
	2	No	634	2.3	0.4	NS
		Yes	149	2.3	0.4	
	3	No	374	2.9	0.3	NS
		Yes	110	2.8	0.3	
>3	0	No	220	2.8	0.3	NS
		Yes	69	2.9	0.2	
	1	No	223	2.8	0.3	NS
		Yes	74	2.7	0.4	
	2	No	337	2.8	0.3	NS
		Yes	121	2.8	0.3	
	3	No	446	2.9	0.3	<0.01
		Yes	229	2.7	0.4	
	>3	No	1994	2.7	0.4	<0.01
		Yes	1147	2.6	0.4	

If there were ≤3 cleavage stage embryos available for transfer but none of good grade, transferring all available embryos was associated with an insignificantly higher probability of pregnancy (8.9% vs 5.3%, p: 0.08). If at least one embryo was of good grade there was no benefit in transferring >2 embryos. If there were >3 cleavage stage embryos available for transfer but none of good grade, then 3-embryo transfer provided an insignificantly higher probability of pregnancy than <3-embryo transfer (24.6% vs 18.2%, p: 0.28). If at least one of the embryos was of good grade there was no benefit in transferring >2 embryos (Table 3.25).

Table 3.25: Cleavage Stage and Good Grade Embryos for the Number of Embryos Transferred (Categorical Variable)

Number of			Number of Embryos Transferred			
Cleavage Stage Embryos	**Good Grade Embryos**	**Ongoing Pregnancy**	**<3**	**3**	**Pregnancy Rate**	**p**
≤3	0	No	94.7%	91.1%	6.3%	NS
		Yes	5.3%	8.9%		
	1	No	87.5%	85.9%	12.8%	NS
		Yes	12.5%	14.1%		
	2	No	80.8%	81.3%	19.0%	NS
		Yes	19.2%	18.7%		
	3	No	71.2%	78.0%	22.7%	NS
		Yes	28.8%	22.0%		
>3	0	No	81.8%	75.4%	23.9%	NS
		Yes	18.2%	24.6%		
	1	No	61.9%	77.3%	24.9%	<0.05
		Yes	38.1%	22.7%		
	2	No	70.4%	74.2%	26.4%	NS
		Yes	29.6%	25.8%		
	3	No	46.4%	69.4%	33.9%	<0.01
		Yes	53.6%	30.6%		
	>3	No	55.6%	66.4%	36.5%	<0.01
		Yes	44.4%	33.6%		

3.8 Prognostic Significance of Number of Embryos Transferred for Ongoing Pregnancy with Consideration of Patient Characteristics and Variables of IVF (Fertilisation)

When the information for embryo quality is not available for assessment, clinical surrogate markers can be used to define a good prognostic subgroup where the number of embryos transferred is no longer an influential factor for the outcome. In the following section, patient characteristics and variables of *in vitro* fertilisation were used as surrogate markers for embryo quality.

The prognostic value for the prediction of ongoing pregnancy of different cut-off levels of female age, duration of subfertility and number of cleavage stage embryos was calculated using a series of ROC curve analyses. Female age at 35 years had a sensitivity of 40-51%, at 38 years 15-21%, and at 40 years 5-9%. Duration of subfertility at four years had a sensitivity of 45-61% and at eight

years 12-17%. Three cleavage stage embryos had a sensitivity of 85-99%, five 55-72% and 10 a sensitivity of 6-11% *(Table 3.54-3.56CD).*

These cut-off levels selected by the ROC curve analysis for female age (35, 38, 40 years), duration of subfertility (4, 8 years), and number of cleavage stage embryos (3, 5, 10) were applied to the whole study population to define the sub-groups. Ongoing pregnancy rates differed significantly between the sub-groups *(Table 3.57-3.64CD).* Hence, these variables were used as surrogate markers with prognostic significance to evaluate the importance of the number of embryos transferred.

When the whole study population was evaluated, the number of embryos transferred was significantly different between the pregnant and non-pregnant groups. The mean number of embryos transferred in the pregnant group was 2.68 and in the non-pregnant group 2.55. In the pregnant group, 69.1% of ETs were 3-embryo transfers as opposed to 63.7% in the non-pregnant group (Table 3.26, 3.27). Next, the study population was evaluated by sub-group analysis.

Table 3.26: Number of Embryos Transferred for Ongoing Pregnancy (Continuous Variable)

		Number of Embryos Transferred		p
Ongoing Pregnancy	No	N	7180	<0.01
		Mean	2.5	
		Median	3.0	
		SD	0.7	
	Yes	N	2475	
		Mean	2.6	
		Median	3.0	
		SD	0.5	

Table 3.27: Number of Embryos Transferred for Ongoing Pregnancy (Categorical Variable)

		Number of Embryos Transferred	N	Percent
Ongoing Pregnancy	No	1	846	11.8
		2	1756	24.5
		3	4578	63.7
	Yes	1	71	2.9
		2	692	28.0
		3	1712	69.1

Number of embryos transferred was still significantly higher in the pregnant group when compared with the non-pregnant group in all sub-groups defined by female age (≤35 or >35 years; ≤38 or >38 years; ≤40 or >40 years, *Table 3.65-3.67CD),* duration of subfertility (≤4 or >4 years; ≤ 8 or >8 years, *Table 3.68, 3.69CD)* and all their combinations (female age <35 years with a duration of subfertility <4 years; <35 years with a duration of subfertility >4 years; >35 years with a duration of subfertility <4; >35 years with a duration of >4 years, *Table 3.70CD*). This was also true for women ≤38 or >38 years; ≤40 or >40 years with a duration of subfertility: ≤4 or >4 years; or ≤ 8 or >8 years *(Table 3.71-3.75CD).*

Therefore, female age and duration of subfertility, either alone or in combination, failed to identify a sub-population where the probability of ongoing pregnancy is independent of the number of embryos transferred. Numerical embryological data were then incorporated into the analysis.

In couples with ≤3 cleavage stage embryos, a greater number of embryos were transferred in the pregnant group when compared with the non-pregnant group (2.3 vs 2.1; p: <0.001); with >3 embryos, fewer embryos were transferred in the pregnant group compared with the non-pregnant group (2.7 vs 2.8; p: <0.001, *Table 3.76CD).*

As the cut-off level increased from 3 to 12 cleavage stage embryos available for ET, a similar trend was maintained *(Table 3.77-3.81CD).* Furthermore, as the cut-off levels increased, the probability of pregnancy improved for couples with a greater number of embryos created and the number of embryos required for pregnancy was progressively reduced (2.7 for 3 cleavage stage embryos to 2.5 for 12 cleavage stage embryos) *(Table 3.76-3.81CD).* Nevertheless, a cut-off number of embryos as low as three showed significant differences between the pregnant and non-pregnant groups.

Although statistically significant, small differences at decimal level in the mean number of embryos transferred is not helpful in clinical practice. Therefore, the significance of number of embryos transferred in the prediction of ongoing pregnancy was reanalysed using categorical variables of <3- or 3-embryo

transfer. Female age, duration of subfertility and the number of cleavage stage embryos were used as surrogate clinical markers of embryo quality.

In poor prognostic groups (women with a lower number of cleavage stage embryos available for transfer), transferring 3 embryos persistently provided higher ongoing pregnancy rates than transferring <3 embryos. Furthermore, regardless of whether 3 or <3 embryos were transferred, pregnancy rates increased progressively with increasing availability of cleavage stage embryos. Although, a 5% difference in pregnancy rates was maintained between 3 and <3 embryos transferred with up to 9 cleavage stage embryos available, the impact of higher numbers of embryos transferred become less obvious (Table 3.28).

In good prognostic groups (women with a higher number of cleavage stage embryos available), transfer of <3 embryos persistently provided higher ongoing pregnancy rates than 3-embryo transfer. Furthermore, regardless of 3- or <3-embryo transfer, pregnancy rates did not alter appreciably with increasing availability of cleavage stage embryos. At cut-off levels up to 9 cleavage stage embryos created, with <3-embryo transfer, the ongoing pregnancy rate was maintained at 42-43% and with 3-embryo transfer at 31-34% (Table 3.29).

Table 3.28: Number of Embryos Transferred in Sub-groups of Cleavage Stage Embryos in the Poor Prognostic Group for Ongoing Pregnancy

Number of		Ongoing Pregnancy		p
Cleavage Stage Embryos	Embryos Transferred	No	Yes	
≤12	<3	75.4%	24.6%	<0.01
	3	72.1%	27.9%	
≤10	<3	76.0%	24.0%	<0.01
	3	72.2%	27.8%	
≤9	<3	76.6%	23.4%	<0.01
	3	72.5%	27.5%	
≤6	<3	80.4%	19.6%	<0.01
	3	75.0%	25.0%	
≤3	<3	86.8%	13.2%	<0.01
	3	82.2%	17.8%	

Table 3.29: Number of Embryos Transferred in Sub-groups of Cleavage Stage Embryos in the Good Prognostic Group for Ongoing Pregnancy

Number of		Ongoing Pregnancy		p
Cleavage Stage Embryos	Embryos Transferred	No	Yes	
>12	<3	64.6%	35.4%	NS
	3	66.1%	33.9%	
>10	<3	59.9%	40.1%	NS
	3	67.9%	32.1%	
>9	<3	58.5%	41.5%	<0.05
	3	66.5%	33.5%	
>6	<3	57.8%	42.2%	<0.01
	3	66.1%	33.9%	
>3	<3	56.8%	43.2%	<0.01
	3	69.0%	31.0%	

3.9 Univariate Analysis of Patient Characteristics and Variables of IVF (Ovarian Stimulation) for Number of Embryos Implanted

Clinical and embryological parameters were evaluated for their distribution in four outcome categories defined as no-implantation, and single, two and more than two gestational sacs detected at the time of first ultrasonographic assessment following positive pregnancy test (Table 3.30).

Among the four outcome groups, the following variables differed significantly: female age, early follicular phase FSH concentration, gravidity, duration of subfertility, number of previous IVF cycles, total gonadotrophin dose, duration of ovarian stimulation, number of developing follicles, endometrial thickness, number of oocytes collected, number of 2-PN embryos created, number of embryos transferred, and number of embryos cryopreserved (Table 3.31).

There was a significant variation in female age, the number of oocytes collected, and 2-PN embryos created when the no-implantation group was compared with the singleton implantation group, and the singleton implantation group with the multiple implantation groups. The difference between the multiple implantation groups was not significant *(Table 3.82CD).*

The difference between the number of embryos transferred was significant when the no-implantation group was compared with the singleton implantation

group and the singleton implantation group with the multiple implantation groups. The difference between the multiple implantation groups was also significant *(Table 3.82CD).*

Table 3.30: Frequency Distribution of the Number of Embryos Implanted

Number of Embryos Implanted	Frequency	Percent
0	6658	68.6
1	2018	20.8
2	853	8.8
3	168	1.7
4	3	0.0

Table 3.31: Distribution of the Clinical Variables among the Number of Embryos Implanted

	Embryos Implanted	N	Mean ± SD	p
Female Age (year)	0	6659	35.6 ± 5.0	<0.01
	1	2019	34.3 ± 4.4	
	2	853	33.1 ± 3.8	
	≥3	171	32.8 ± 4.0	
Oocytes Collected	0	6605	9.7 ± 5.8	<0.01
	1	2019	10.8 ± 5.8	
	2	853	12.2 ± 5.5	
	≥3	171	12.2 ± 5.5	
Previous IVF Cycles	0	6659	1.9 ± 1.4	<0.01
	1	2019	1.7 ± 1.2	
	2	853	1.6 ± 1.0	
	≥3	171	1.8 ± 1.1	
Duration of Subfertility (year)	0	5203	5.4 ± 3.7	<0.01
	1	1560	4.9 ± 3.3	
	2	705	4.6 ± 3.1	
	≥3	142	4.9 ± 2.9	
Previous Pregnancies	0	6659	0.6 ± 1.1	0.04
	1	2019	0.6 ± 1.0	
	2	853	0.7 ± 1.1	
	≥3	171	0.7 ± 1.2	
Total Gonadotrophin Dose (IU)	0	5154	2925 ± 1262	<0.01
	1	1558	2633 ± 1537	
	2	731	2425 ± 922	
	≥3	142	2448 ± 999	
Duration of Ovarian Stimulation (day)	0	5147	11.0 ± 3.1	0.05
	1	1558	10.9 ± 2.8	
	2	733	11.3 ± 3.3	
	≥3	140	11.9 ± 4.4	

		Embryos Implanted	N	Mean ± SD	p
Number of Follicles Measuring	8-12 mm	0	5173	3.3 ± 2.9	<0.01
		1	1566	3.6 ± 3.0	
		2	737	4.2 ± 3.2	
		≥3	142	4.3 ± 3.3	
	13-16 mm	0	5173	4.0 ± 2.9	<0.01
		1	1566	4.7 ± 3.3	
		2	737	5.0 ± 3.1	
		≥3	142	5.2 ± 2.9	
	17-19 mm	0	5173	3.7 ± 2.6	<0.01
		1	1566	4.1 ± 2.7	
		2	737	4.5 ± 2.8	
		≥3	142	4.6 ± 2.9	
	≥ 20 mm	0	3596	1.8 ± 2.2	NS
		1	1089	1.9 ± 2.3	
		2	521	2.0 ± 2.0	
		≥3	87	1.5 ± 1.4	
Endometrial Thickness (mm)		0	5097	11.0 ± 1.7	<0.01
		1	1544	11.3 ± 1.7	
		2	728	11.4 ± 1.7	
		≥3	141	11.5 ± 1.9	
hCG Day E_2 (nmol/l)		0	2548	6.09 ± 4.57	NS
		1	782	5.90 ± 3.41	
		2	394	6.41 ± 3.38	
		≥3	57	6.24 ± 3.34	
Pre stimulation FSH (IU/l)		0	2642	6.6 ± 3.4	<0.01
		1	801	6.4 ± 2.9	
		2	370	6.0 ± 2.5	
		≥3	68	5.8 ± 2.7	
2-PN Embryos		0	6605	5.1 ± 3.7	<0.01
		1	2019	6.2 ± 3.8	
		2	853	7.4 ± 3.9	
		≥3	171	7.9 ± 3.5	
Number of Embryos Transferred		0	6605	2.5 ± 0.7	<0.01
		1	2019	2.6 ± 0.6	
		2	853	2.7 ± 0.4	
		≥3	171	2.9 ± 0.2	
Number of Embryos Cryopreserved		0	6605	1.0 ± 2.5	<0.01
		1	2019	1.5 ± 3.1	
		2	853	2.4 ± 3.6	
		≥3	171	2.7 ± 3.5	

	Embryos Implanted	N	Mean ± SD	p
Early Follicular Phase Pre-stimulation E_2 (nmol/l)	0	1529	0.23 ± 0.36	NS
	1	470	0.23 ± 0.52	
	2	224	0.28 ± 1.21	
	≥3	38	0.21 ± 0.18	

Both singleton and twin implantations were reduced in cycles complicated by ovarian cysts after down-regulation but the differences did not reach significance (p: 0.06). Both singleton and twin implantations were reduced in cycles complicated by sonographically detectable hydrosalpinx but the differences did not reach significance (p: 0.3) *(Table 3.83, 3.84CD).*

Of all singleton deliveries, 59% had one, 34% had two and 7% had three embryos implanted. Of the twin deliveries, 80% had two and 20% had three embryos implanted. Of the triplet deliveries, 98% had three and 2% had four embryos implanted *(Table 3.85-3.87CD).*

3.9.1 Univariate Analysis of Patient Characteristics and Variables of IVF (Transferred-Embryo Quality) for Singleton vs Multiple Pregnancy

There were significant differences when the following variables were compared between singleton and multiple pregnancies respectively: Female age (34.3 vs 33.1 years), duration of subfertility (5 vs 4.6 years), number of oocytes collected (10.8 vs 12.2), number of 2-PN embryos created (6.2 vs 7.5), number of embryos transferred (2.6 vs 2.8), grade of the first (1.7 vs 1.5), second (1.9 vs 1.7) and third (2 vs 1.9) embryos transferred, stage of the second (4.5 vs 4.7) and third (4 vs 4.4) embryos transferred, and number of cleavage stage embryos created (5.6 vs 6.7) *(Table 3.88CD).*

3.9.2 Univariate Analysis of Patient Characteristics and Variables of IVF (Transferred-Embryo Quality) for Singleton vs Twin vs High-order Multiple Pregnancy

The following variables differed significantly when compared between singleton, twin and high order pregnancies respectively; female age (34 vs 33 vs 32 years), number of oocytes collected (10.8 vs 12.2 vs 12.3), number of 2-PN embryos created (6 vs 7 vs 8), number of embryos transferred (2.6 vs 2.7 vs 3), grade of the first (1.7 vs 1.5 vs 1.5), second (1.9 vs 1.7 vs 1.6) and third (2 vs

1.9 vs 1.8) embryo transferred, stage of the second (4.5 vs 4.7 vs 5) and third (4 vs 4.3 vs 4.7) embryo transferred, and number of cleavage stage embryos created (5.6 vs 6.7 vs 7) *(Table 3.89CD).* None of the observed differences in these variables between twin and high order pregnancies were significant, except the number of embryos transferred (p:<0.001).

3.9.3 Multivariate Analysis of Patient Characteristics and Variables of IVF (Transferred-Embryo Quality) for Singleton vs Multiple Pregnancy

After excluding women with no pregnancy, logistic regression analysis was performed on 2400 cycles to predict multiple pregnancy by female age, duration of subfertility, number of 2-PN embryos created and number of embryos transferred as independent variables (Table 3.32).

After the effect of female age and number of 2-PN embryos was corrected, the number of embryos transferred remained the most influential variable on the multiplicity of pregnancy, unlike in the prediction of pregnancy where the number of embryos transferred lost significance after correction for other variables.

Table 3.32: Variables in the Equation for Multiple vs Singleton Pregnancy (1)

	Regression Coefficient	SE	OR	p
Age	-0.08	0.01	0.92	<0.01
2-PN Embryos	0.07	0.01	1.07	<0.01
Transferred Embryos	0.64	0.08	1.90	<0.01

Further logistic regression analysis using the forward stepwise conditional entry method was performed on 1579 cycles of ongoing pregnancy to predict multiple pregnancy with a more comprehensive range of independent variables (Table 3.33).

Table 3.33: Independent Variables used in Logistic Regression Analysis

Age	Transferred embryos
Duration of Subfertility	Grade and Stage of the 1st ET
2-PN Embryos	Grade and Stage of the 2nd ET
Cleavage Stage Embryos	Grade and Stage of the 3rd ET

After the effect of embryo quality, number of cleavage stage embryos and female age were corrected the number of embryos transferred remained as the most significant predictor of multiple pregnancy (Table 3.34).

Table 3.34: Variables in the Equation for Multiple vs Singleton Pregnancy (2)

	Regression Coefficient	SE	OR	p
Age	-0.09	0.01	0.91	<0.01
Transferred Embryos	0.99	0.18	2.70	<0.01
Grade of the 1st ET	-0.28	0.10	0.75	<0.01
Grade of the 2nd ET	-0.31	0.09	0.73	<0.01
Stage of the 3rd ET	0.09	0.03	1.09	0.01
Cleavage Stage Embryos	0.07	0.02	1.07	<0.01

4 Factors Affecting IVF Outcome: Discussion

Using univariate analysis, clinical and embryological variables of IVF cycles with prognostic significance in the probability of ongoing pregnancy and multiple pregnancy were identified. The list of variables was then narrowed by logistic regression analysis to include only the variables with the highest prognostic significance and clinical relevance. Different cut-off levels for these short-listed variables were then tested by ROC curve analysis for their diagnostic power. Using these cut-off levels, the study population was divided into sub-populations. The prognostic significance of the number of embryos transferred was tested by comparing the number of embryos transferred in each sub-population.

4.1 Analysis of Patient Characteristics and Clinical Variables of IVF (Ovarian Stimulation) for Ongoing Pregnancy

The treatment outcome in IVF cycles was closely associated with the response of the ovaries to gonadotrophin stimulation and this was evident in both the pre-stimulation surrogate markers of ovarian reserve and the variables of actual stimulation.

Younger women with a shorter duration of subfertility and fewer previous IVF attempts were more likely to achieve ongoing pregnancy (Table 3.4, *Table 3.13CD)*. These findings are in close agreement with those in the published literature.

Female age is cited as the single most important factor determining the outcome of fertility treatment[8,14,15,16,19,20]. Several others also conclude that the chances of successful live births after IVF drop significantly with increased female age[16,17,18]. Van Kooij et. al.[22] reports a steeper decline after the age of 37 years. Stolwijk et. al.[23] reports a simple linear effect.

It is well established that increased duration of fertility delay is associated with a reduced possibility of conception in both treated and untreated subfertile couples even after adjustment for female age[8].

A large study using the HFEA IVF database reports that the live birth rate per treatment cycle is highest in the first cycle and decreases significantly with increasing numbers of previous treatment cycles, after adjusting for female age. The same finding is reported by the French Registry on IVF[42]. However, Croucher et. al.[20] report that the chance of pregnancy after a single IVF treatment cycle remains constant for the first three attempts and only subsequently begins to decline. Likewise, smaller studies report a constant rise in the cumulative success rate up to six cycles[46,48].

Lower pre-stimulation FSH values were observed in ongoing pregnancy when compared with the non-pregnant and no-ET groups. More importantly, in these outcome groups, FSH levels were well below the traditionally accepted upper limit of 10 IU/l for good prognosis (Table 3.4, *Table 3.13CD*). This implies a dose response relationship between ovarian reserve and the treatment outcome. This is valid even when the rise in FSH levels occurs within the normal range[66,67,68]. Basal serum FSH levels reflect the number of antral follicles ready for recruitment[62] and rising levels of early follicular phase FSH reveal a reduction in the size of this follicular cohort[30,31,36], with fewer follicles developed, fewer oocytes collected, and fewer embryos created in response to gonadotrophin stimulation[63,64,65,67,69,85,86].

Likewise, cycles with ongoing pregnancy were characterised by higher levels of ovarian response than non-conception cycles. This relationship also manifested in a dose response pattern, whereby the poorest response was usually associated with the highest probability of no-ET. The treatment cycles that produced more follicles and oocytes with less intensive gonadotrophin stimulation and higher E_2 concentrations with thicker endometrium before hCG injection, were more likely to have a successful outcome. A functional association between qualitative and quantitative ovarian response became evident with the high E_2 levels linked to the high number of follicular development and subsequently to the collection of a greater number of oocytes and the creation of a greater number of embryos. Hence the women who had a higher number of embryos had a higher chance of pregnancy. In the pregnant group, a thicker endometrium also reflected the presence of a responsive end

organ and exemplified the crucial link between ovarian and endometrial synchrony for implantation (Table 3.4, *Table 3.13CD*).

In subgroup analyses, the distribution of these variables in different pregnancy outcomes was evaluated (*Table 3.16CD*). The women who achieved ongoing pregnancy were significantly younger than those in the no-ET, no-pregnancy, or miscarriage group, but not younger than women who had ectopic pregnancy or biochemical pregnancy. This emphasised the age effect over ovarian reserve and ovarian response capacity in the genesis of pregnancy and miscarriage. To exemplify this contention more precisely, in cycles with ongoing pregnancy the number of oocytes collected was significantly higher than in miscarriage, no-pregnancy and no-ET cycles, but not higher than cycles leading to ectopic or biochemical pregnancy. The same trend was maintained for duration of subfertility, total gonadotrophin dose required for ovarian stimulation, and number of embryos created and cryopreserved. All of these variables complied with a common theme that better ovarian response leads to less miscarriages and better ongoing pregnancy rates. Although the pre-stimulation FSH levels of women who would achieve ongoing pregnancy was significantly lower than in the no-ET or no-pregnancy women, there was no significant difference in women who would miscarry. This manifests in the limitation of surrogate markers to represent the full spectrum of the biological processes. When the contrast between the outcomes was distinct, as in the case of the dichotomous outcomes of pregnant vs non-pregnant, surrogate markers like FSH tended to function better. However, when the difference between outcomes was gradual over a wide transition, as in the case of biochemical pregnancy, miscarriage, and ongoing pregnancy, the discriminative power of FSH did not reach a level of significance (*Table 3.16CD*).

Endometrial thickness measured on the day of hCG injection was significantly higher in women with ongoing pregnancy than in miscarriage, no-pregnancy and no-ET cycles. However, the measurements were very similar, within fractions of a millimetre. Therefore, despite emphasising the key role of physical development in endometrial receptivity, the clinical application of endometrial thickness is limited for practical reasons. It is of note that the mean endometrial

thickness in ongoing intrauterine pregnancy and ectopic pregnancy was similar, and so this clinical observation alone should not be used for differential diagnosis *(Table 3.16CD).*

Sonographically visible hydrosalpinx decreased the ongoing pregnancy rate without affecting the prevalence of no-ET cycles. Furthermore, hydrosalpinx doubled the incidence of biochemical pregnancy but did not alter the probability of miscarriage *(Table 3.15, 3.18CD).* Tubal factor itself did not affect the biochemical pregnancy rate. Hence hydrosalpinx should present a unique toxic effect on implantation. This may provide circumstantial evidence that its toxic effect is dose dependent and likely to affect implantation with premature termination rather than through the ovarian response[494]. Somewhat unexpectedly, no ectopic pregnancy was observed in this group.

The presence of ovarian cysts following down-regulation significantly reduced the chances of ongoing pregnancy and was associated with a higher probability of no-ET. Ovarian cysts may exert this negative effect either through altering the paracrine milieu in the ovary, thereby suppressing follicular recruitment and maturity, or by exerting a space occupying effect on the remaining ovarian tissue and physically restraining its response to stimulation *(Table 3.14, 3.17CD).*

The number of embryos transferred had an influence on the chances of pregnancy (this will be detailed in the following sections), but no prognostic determination was noted over the outcome of pregnancy in terms of miscarriage or biochemical or ectopic pregnancy (Table 3.4, *Table 3.13, 3.16CD).*

Following these univariate analyses, logistic regression analysis was performed on 2109 cycles to predict the independent prognosticators of ongoing pregnancy. A wide range of clinical variables was studied, including the demographic characteristics of couples, details of ovarian stimulation and ovarian response. Female age, duration of subfertility, total gonadotrophin dose, number of oocytes collected, number of 2-PN embryos created, and number of

embryos transferred were found to be significant for ongoing pregnancy (Table 3.5).

The univariate and multivariate analyses revealed the same finding by emphasising the pivotal role of ovarian reserve (female age), severity of the pathology behind subfertility (duration of subfertility) and the level of ovarian response, for determining pregnancy. However, logistic regression analysis has shown that pre-stimulation FSH levels, E_2 concentrations and endometrial thickness before hCG injection, aetiology of subfertility, previous IVF attempts, presence of hydrosalpinx or ovarian cysts, number of ovarian follicles and cryopreserved embryos lost their prognostic significance once their confounding effects were corrected against each other. This is because different clinical variables can surrogate the same underlying physiological process but with varying levels of association. Regression analyses tend to select those with the highest level of association.

4.2 Factors Related to Oocyte Maturity

The clinical variables characterising the demographic features of the patients and the response parameters of the ovarian stimulation were analysed in 12,332 cycles to establish the most informative surrogate markers of oocyte quality (Table 3.6, 3.7, *Table 3.19-3.26CD).*

The number of mature oocytes correlated negatively with both female age and early follicular phase FSH concentration, confirming the clinical value of these two most commonly employed surrogate variables in the prediction of ovarian reserve. The number of mature oocytes was also positively associated with the number of previous pregnancies. This observation not only highlighted the clinical value of the obstetric history as a surrogate variable for the current probability of pregnancy, but also provided circumstantial evidence for the presence of a functional quality shared by the collectable oocytes in the ovary.

The number of mature oocytes increased with the total number of collected oocytes. This was not just a simple mathematical equation but also highlighted the positive correlation between qualitative and quantitative ovarian response to

stimulation. This contention found further support in the observed positive correlation of the number of mature oocytes to the number of developing follicles and E_2 level before hCG injection. Further evidence of the association between optimum follicular size and oocyte maturity, was the observed correlation coefficient of follicles measuring 13-19mm being twice that of follicles measuring <13mm and six times that of follicles measuring >20mm. Hence, follicular development to >20mm should not be encouraged to optimise oocyte maturity. Moreover, a negative correlation of the number of mature oocytes with immature oocytes probably reflects the biological tendency of ovaries to support one dominant cohort of growth.

It is likely that the potential to provide mature oocytes on stimulation is an intrinsic response capability of the ovaries. The number of mature oocytes correlated negatively with the total gonadotrophin dose required for ovarian stimulation and with the duration of stimulation. Although quantitative response may be augmented by utilising more aggressive stimulation protocols this is less likely to improve the qualitative response.

The literature provides supporting evidence for this contention in reporting that a low number of developing follicles is associated with poor oocyte and embryo quality[495] and reduced embryo viability[496]. In a study of poor responders, no improvement is reported in any of the stimulation parameters when six ampoules of FSH were used instead of four[497]. In a prospective randomized study, van-Hooff et. al.[498] report that increasing the daily gonadotrophin dose to >225 IU fails to improve the ovarian response in poor responders. Similar studies comparing recombinant FSH 100 IU vs 200 IU[499,500] and 150 IU vs 200 IU[501] report that pregnancy rates are not influenced by gonadotrophin doses. In a group of woman with a first cycle abandoned due to poor response, increased gonadotrophin dosage does not appear to improve outcome in subsequent treatment[502]. While a large number of ampoules can marginally increase the probability of collecting oocytes, this might also be associated with decreased probability of fertilization and of implantation of the resulting embryos[19]. A detrimental effect of high dose gonadotrophin stimulation in relation to the zona pellucida thickness is suggested by Bertrand et. al.[503] who conclude that a small

increase in the number of oocytes collected with a high-dose regimen is not translated into any other quality improvement in embryological variables.

The number of mature oocytes was linked to better treatment outcome and correlated positively with the number of 2-PN embryos created, number of embryos cryopreserved and the number of embryos implanted (Table 3.7).

Because the number of mature oocytes is a function of the total number of oocytes collected, the same analyses were repeated with the percentage of mature oocytes. In mathematical terms, this parameter was independent of the total numbers. The results revealed the same associations, with the exception of E_2 levels on the day of hCG administration, which lost its association with the percentage of mature oocytes. However, serum E_2 levels continued to correlate with the percentage of immature follicles. Hence, serum E_2 level was not a reliable surrogate marker for high oocyte quality. The positive link between the percentage of immature oocytes and E_2 levels also explains the positive association observed between the percentage of immature oocytes and the endometrial thickness, which was missing in the case of mature oocytes.

The percentage of immature oocytes correlated positively with the total gonadotrophin dose and the duration of ovarian stimulation, but mature oocytes did not. Hence, this is a further verification that employing aggressive stimulation regimens will mostly help to increase the immature but not the mature oocyte response.

Unlike mature oocytes, the implantation potential of embryos created by immature oocytes was significantly lower. This contention was supported by a negative association with the number of implanted embryos and the percentage of immature oocytes, as opposed to a positive association with the percentage of mature oocytes. Therefore, a negative association between higher levels of gonadotrophin stimulation and the chances of implantation and pregnancy is apparent.

The percentage of post-mature oocytes correlated positively with female age and early follicular phase FSH concentration. Accelerated follicular development and oocyte maturation occurs in older women, due to higher early follicular phase FSH concentrations. This was not unique to spontaneous cycles but was also observed in stimulated cycles. Replacing the role of high endogenous FSH concentration, the intensity of gonadotrophin stimulation was found to be associated with the post-maturity of oocytes. The number of post-mature oocytes correlated positively with the number of developing follicles, particularly those measuring >20mm. Furthermore, despite a positive correlation with the number of embryos created, post-mature oocytes did not associate with the number of implanted embryos, indicating a deterioration of oocyte quality in terms of implantation potential with increasing follicular size and post-maturity.

In women with PCOS, the percentage of mature oocytes was similar to that of non-PCOS women but the percentage of immature and post-mature oocytes was higher, at the expense of borderline mature oocytes. Hence, in women with PCOS, the extremes of oocyte maturity, either delayed or accelerated, was likely to occur during ovarian stimulation. Unlike common expectations, only a clinically insignificant increase in the number of oocytes collected was observed in PCOS women *(Table 3.21CD)*.

The systemic endocrine environment during the later stages of follicle development is shown to have a crucial role in coordinating follicular and oocyte maturation before ovulation[504]. Despite PCOS being associated with abnormal circulating hormones, abnormal peri-follicular vascularity and significant abnormalities of granulosa cell function, after *in vitro* maturation of oocytes or following ovulation induction for IVF, oocyte and embryo quality are not obviously impaired in PCOS[504]. These findings are in agreement with those presented in the current study. In contrast, Cano et. al.[505] show that there is a particular subgroup of PCO patients with lower fertilization rates and embryos unable to implant. These patients are obese and non-hyperandrogenic and show derangements of insulin secretion. The presented data did not allow a subgroup analysis for specific endocrine abnormalities, such as

hyperinsulinaemia, and this may explain the discrepancy between the current study and others.

There was no apparent detrimental effect of endometriosis on the quantitative and qualitative response of the ovaries. The effect of different grades of endometriosis on these variables was not consistent and not clinically significant, with the exception of percentage of oocytes collected. The probability of finding an oocyte in the follicular fluid declined significantly from 88% in mild endometriosis to 83% in severe endometriosis *(Table 3.22, 3.23CD).*

In contrast to the findings of the current study, poor quality of oocytes has been suggested as one possible cause of subfertility[506]; higher apoptotic incidence, more alterations of the cell cycle, and a higher incidence of oxidative stress are among the other possible mechanisms suggested in endometriosis when compared with other subfertility causes[507]. Reliance on history based data collection on aetiology, which may not be as reliable as small scale but well controlled clinical trials, can explain the discrepancy between this study and the others.

In unexplained subfertility, the percentage of mature oocytes was significantly lower and the percentage of post-mature oocytes higher *(Table 3.24CD).* This was the same pattern as that observed in the older women with high FSH values. Hence, one of the possible explanations behind unexplained subfertility is likely to be a subtle ovarian failure with a tendency to accelerated oocyte maturity.

Women who developed ovarian cysts following down-regulation developed a similar number of follicles to those without such baseline cysts, but the number of oocytes collected was less, albeit at a clinically insignificant level. This equates with lower oocyte collection rates in the presence of ovarian cysts. No effect over the level of maturation was observed. Hence, the effect could be a pure space occupying effect, rather than paracrine suppression of ovarian response *(Table 3.25CD).* The effect of hydrosalpinx was insignificant on the

qualitative response of ovaries in terms of oocyte maturity. However, the number of follicles aspirated and oocytes collected was lower, albeit at a clinically insignificant level. Hence, the main site of action is less likely to be the ovaries *(Table 3.26CD).*

4.2.1 Analysis of Variables of IVF (Oocytes) for Ongoing Pregnancy

We have seen that treatment cycles characterised by a greater number of follicles aspirated and oocytes collected were more likely to be those with conception. However, in numerical terms the differences were small, being one or two oocytes at most. The maturity of the oocytes was what appeared to be more important. Conception cycles had a higher percentage of mature oocytes and lower percentage of immature oocytes (Table 3.8). In 7013 cycles, logistic regression analysis evaluating the prognostic significance of the number of mature, borderline, immature, and post-mature oocytes in the prediction of ongoing pregnancy, revealed that it was the number of mature and borderline mature oocytes that possessed the prognostic significance (Table 3.9).

Degree of oocyte maturity also influenced the probability of miscarriage, biochemical pregnancy and ongoing pregnancy. The percentage of mature oocytes dropped progressively from ongoing pregnancy to miscarriage and biochemical pregnancy. The percentage of mature oocytes was highest in women who eventually developed ectopic pregnancy and lowest in women with no-ET. On the other hand, the percentage of borderline mature and immature oocytes increased from ongoing pregnancy to biochemical pregnancy. The percentage of post-mature oocytes was higher in women who developed biochemical pregnancy *(Table 3.27CD).*

These associations provided evidence for the contention that biochemical pregnancy, miscarriage and ongoing pregnancy are likely to be the progressive stages in the process of implantation. In this section this argument was furthered by emphasising the role of oocyte maturity as the underlying factor determining the ability of embryos to proceed through the stages of implantation; the greater the maturity of the oocyte, the greater the probability of completing the implantation process and establishing ongoing pregnancy. As

oocyte maturity declines, the implantation process is hampered in a dose dependant way. Hence, if maturity is low the outcome will likely to be miscarriage, if lower the outcome will be biochemical pregnancy, and if lowest the outcome will be no embryo transfer. The observation that the highest percentage of oocyte maturity was in the cycles resulting in ectopic pregnancy raises an interesting hypothesis; that the resulting embryos had the highest implantation potential and so became less selective of the receptivity of the implantation site.

4.3 Analysis of Variables of IVF (Fertilisation, Individual Embryo Quality) for Ongoing Pregnancy

Embryo quality is a term used in clinical practice to define the development potential of embryos *in vitro*. The estimation of embryo quality is used so that the best embryos can be selected for transfer to enhance the probability of implantation[19,22,23,104]. The morphological features of the dividing embryo are well documented prognostic factors in determining the treatment outcome[16,92,104,105,106,115,116,130,508,509,510].

To evaluate the prognostic value of embryo quality in the treatment outcome, 8090 treatment cycles were studied. The embryos were anatomically graded from 1 for the best to 6 for the worst. The blastomeres were counted and graded as a developmental stage. The combination of these two variables was documented as S()G() for all created embryos. Of the collected oocytes, 68% were fertilised and of these 82% reached the cleavage stage (Table 3.10, 3.11).

It was demonstrated that the number of oocytes used for fertilisation, number of oocytes fertilised and number of embryos achieving cleavage stage was significantly higher in conception cycles. Despite fertilisation rates (2PN embryos/oocytes used for fertilisation) being higher in conception cycles, the cleavage rate of the fertilised embryos (cleavage stage embryos/2PN embryos) did not differ between conception and non-conception cycles in the univariate analysis (Table 3.12). To evaluate this unexpected finding, logistic regression analysis was performed in 3616 treatment cycles using the number of oocytes used for fertilisation, number of oocytes fertilised, number of cleavage stage

embryos created, fertilisation rate, cleavage rate and number of embryos in different quality groups within the cohort as independent predictors of the treatment outcome. This revealed that the number of oocytes used for fertilisation, fertilisation rate and cleavage rate positively affected the outcome. Unlike univariate analysis, regression analysis designated the percentage of cleavage stage embryos (cleavage rate) as a significant prognosticator. Hence the percentage of first mitotic division in the whole embryo cohort is discriminative for outcome (Table 3.13).

Nevertheless, the fertilisation rate of the oocytes had the highest predictive value among all qualitative and quantitative variables tested in this analysis (Table 3.13). This is in contrast to the published literature, in which the prognostic importance given to fertilisation and cleavage rates is not high[71,75,76,77,511,512]. It is proposed that even those oocytes collected from women with poor ovarian reserve can undergo fertilisation and cleavage and produce high quality embryos[76]. However, the implanting ability of such embryos declines with female age and thus pregnancy rates decline[75,76,79]. It is argued that chromosomal abnormalities of the oocyte do not need to affect fertilization and early embryo cleavage, but can be linked to failure of implantation[80,81,82]. In contrast to the published literature from smaller study populations, the statistical power of the current study allowed the detection of differences that may have otherwise failed to reach significance.

Higher numbers of oocytes and embryos favouring a successful outcome indicated the opportunity of better selection from a larger cohort. The probability of finding good quality embryos would be higher, for simple mathematical reasons, if the selection involved a greater number of candidates. However, it was also the percentage of fertilised oocytes that affected the probability of pregnancy. Hence, an intrinsic quality response linked to the quantitative response was also noticeable, indicating that it was not only the size of the cohort that improved the probability of finding a good quality embryo but also the inherent ability of larger cohorts to develop better quality embryos, thereby improving the probability of pregnancy.

Different expression (% or numbers) of the same variable may selectively highlight the relative importance of the quantitative and qualitative aspects of the ovarian response for their surrogate roles in future embryonic development and implantation. In this setting, numbers were regarded as surrogate markers for quantitative and percentages for qualitative response.

Both the size of the cohort in terms of available oocytes, and the quality in terms of ability to achieve higher fertilisation and cleavage rates, independently affected the probability of pregnancy. Furthermore, irrespective of the morphological appearance of the developing blastomeres, slow cellular division indicated a severe developmental problem leading to a low probability of pregnancy. On the other hand, a rapidly dividing embryo did not necessarily possess high implantation potential, unless the developing blastomeres were morphologically of good quality. Four-cell stage embryos with good grade development had the best probability of pregnancy. This highlighted an optimum developmental stage that maximises the probability of further development in utero and the probability of implantation and pregnancy (Table 3.13).

The number of blastomeres at the time of transfer has been used as a proxy measure of the development potential of the embryo, linking closely to the embryonic genome activation that occurs between the four-cell and eight-cell stages of pre-implantation development[125]. Erenus et. al.[104] report that embryos of at least two mitotic divisions implant better than two-cell embryos of comparable morphology and that heavily fragmented embryos do not implant as well as embryos with fewer or no anucleate fragments. Giorgetti et. al.[116] and Ziebe et. al.[126] report that the transfer of four-cell embryos results in a higher implantation and pregnancy rate when compared with two-cell and three-cell embryos. Furthermore, the transfer of four-cell embryos results in a significantly higher pregnancy rate compared with embryos cleaved beyond the four-cell stage. Fast cleavage with more than five cells on day 2 is unfavourable, especially if associated with ≥10% fragmentation, as the probability of chromosomal abnormality appears to be higher[513].

The morphological development of the individual blastomeres became decisive for the outcome only if the embryo exhibited good growth in terms of cellular division. A poor developmental stage with few blastomeres was not compensated by superior morphology. The association between the grading of the embryos and their developmental stage in terms of number of blastomeres was weak and likely to reflect both grading and staging operate independently in the probability of implantation. Good grade embryos and those with a greater number of blastomeres had higher implantation potential *(Table 3.35-3.37CD).*

After assessment of the quality of the individual embryos, the importance of the global quality of the whole embryo cohort in determining the treatment outcome was also appraised. Grade 1 and 2 embryos, classified as 'good grade' regardless of their developmental stage, dominated the embryo cohort in women who went on to achieve pregnancy (Table 3.14).

4.4 Analysis of Variables of IVF (Quality of the Embryos Transferred) for Ongoing Pregnancy

After confirming the importance of quality in the whole embryo cohort created in the same stimulation cycle, the impact of quality of the best three embryos selected for transfer was investigated. Univariate analysis revealed that both stage and grade of the embryos transferred were prognostic for ongoing pregnancy *(Table 3.38CD).*

The multivariate analysis used patient characteristics (female age, duration of subfertility, number of previous IVF attempts, FSH level), details of the ovarian stimulation (total dose of gonadotrophin, duration of ovarian stimulation, hCG day E_2), and embryological variables (number of oocytes collected, and number of mature oocytes, borderline oocytes, 2-PN and cleavage stage embryos, number of embryos cryopreserved, quality of the individual embryos transferred, and quality of the individual embryos in the whole embryo cohort). The number of embryos transferred was used as a constant in the equations to maintain stability in its effect over the outcome.

Female age, number of follicles, quality of the embryos transferred and quality of the individual embryos in the whole cohort were prognostic. The quality of the individual embryos in the whole cohort had the highest impact on the outcome (Table 3.20). Therefore, the quality of untransferred sibling embryos affected the probability of pregnancy independently of the quality of the embryos transferred. This indicated that a common characteristic inherently present in the embryo cohort determined their growth potential.

Wheeler et. al.[514] construct a similar statistical model and conclude that the probability of pregnancy increases with an increasing total embryo score but decreases with increasing female age. Hunault et. al.[515] demonstrate that the best predictors of ongoing pregnancy after multivariate analyses are the number of oocytes collected, the development stage of the second-best embryo transferred, and the morphology score of the best embryo transferred.

On the other hand, neither the quality of the best three embryos nor the total number of good grade embryos in the cohort differentiated the outcome in terms of biochemical pregnancy, miscarriage, or ectopic pregnancy, reflecting the presence of other mechanisms operating in their pathogenesis and unrelated to or indefinable by these embryological variables *(Table 3.45CD).* Levy et. al.[44] also demonstrate that morphological grading of embryos is similar in an ongoing or biochemical pregnancy after IVF treatment.

4.4.1 Factors Linked to Embryo Quality

The quality of transferred embryos, semi-quantified in terms of embryo grading and quantified by their blastomere numbers, was strongly associated with the clinical surrogate markers of the ovarian reserve (Table 3.16, *Table 3.35-3.37CD).* Younger women who produced more oocytes with higher levels of E_2 in response to low intensity ovarian stimulation, had better quality embryos. Therefore, it was the inherent ability of the women, not the intensity of the ovarian stimulation that dictated the quality of the embryos. While quantitative ovarian response can be augmented with more aggressive ovarian stimulation regimens, within the limits of ovarian reserve, this is less likely to improve the qualitative outcome. It is of note that the same conclusion was reached in the

section on oocyte quality. This association further highlighted the pivotal role of ovarian reserve and its response capability to stimulation in the creation of high quality embryos with the best probability of implantation. In this context, it was female age but not the pre-stimulation FSH levels that correlated with the quality of transferred embryos.

In the whole cohort, embryo quality varied within a narrow range and shared similar traits. The quality of the best three embryos strongly correlated with each other but also with the size of the cohort. As the number of 2-PN and cleavage stage embryos increased the quality of the embryos transferred also increased. Once again, this was not simply a reflection of a better selection opportunity made possible by the availability of a larger cohort, but also an indication of inherent quality. Women who could produce greater numbers of embryos could also produce better quality embryos *(Table 3.37CD)*.

4.5 Prognostic Significance of Number of Embryos Transferred for Ongoing Pregnancy

The number of embryos transferred was higher in the pregnant group, but once the quality of the individual embryos in the whole cohort, the global quality of the whole embryo cohort or the quality of the embryos transferred was taken into consideration, the number of embryos transferred was no longer a significant determinant of the treatment outcome (Table 3.17, 3.18, 3.21, 3.23). In this section this important observation is further discussed by using surrogate clinical variables for embryo quality.

Female age, duration of subfertility, and the number of cleavage stage embryos created were prognostic variables of IVF treatment. In the current study a pragmatic selection was made for different cut-off levels of each variable (female age: 35, 38, 40 years; duration of subfertility: 4, 8 years; number of available embryos: 3, 5, 10) and their predictive power was evaluated for ongoing pregnancy. Higher cut off levels were associated with progressively increasing specificity but at the expense of dropping sensitivity in the prediction of ongoing pregnancy after IVF treatment. This indicates that higher cut-off levels are good at identifying women who are less likely to achieve pregnancy,

but not at identifying women who are more likely to achieve pregnancy. All cut-off levels significantly demarcated the good and poor prognosis, albeit at varying levels of sensitivity and specificity. Among all three variables, depending upon the cut off level, the number of cleavage stage embryos had the highest sensitivity and specificity in the range of 92 to 99% *(Table 3.56CD).*

These cut-off levels were applied individually and then in combination to create different sub-groups of the whole study population for comparison of the number of embryos required to achieve pregnancy in each category.

Women with a greater number of embryos transferred were more likely to achieve pregnancy, regardless of age ≤35 or >35 years, ≤38 or >38 years, ≤40 or >40 years, or duration of subfertility ≤4 years or >4 years, or ≤8 or >8 years *(Table 3.65-3.75CD).* On the other hand, the number of cleavage stage embryos available for transfer defined a subgroup of women in whom transferring more embryos did not positively affect the treatment outcome *(Table 3.76-3.81CD).* While women with ≤3 cleavage stage embryos still needed a greater number of embryos transferred to improve the probability of pregnancy, women with >3 embryos did not need to increase the number of embryos transferred to achieve pregnancy.

With increasing availability of cleavage stage embryos from 3, pregnancy rates were steeply increased then began to plateau at the 9-embryo level. However, with 3 cleavage stage embryos available for transfer, the difference in pregnancy rates between 3-embryo and <3-embryo transfer was almost the same as that observed with 9 embryos available and this difference changed only marginally with increasing availability of embryos. Hence, the difference in pregnancy rates between 3-embryo and <3-embryo transfer with higher numbers of cleavage stage embryos was mostly due to a difference that originated from the group with 3 embryos available and was then carried through to the higher cut-off levels, which consistently included that group (Table 3.28, 3.29. *Table 3.76-3.81CD*). Therefore, it was concluded that 3-embryo transfer was only beneficial when only 3 embryos were available for transfer and women with >3 embryos did not benefit from high-number ET. This

statement can be applicable as a population based protocol where the details of the individual patients (age, duration of subfertility, and quality of individual or transferred embryos) are pragmatically not used for decision making. However, the guidance can be more refined if the knowledge of embryo quality is also available. It was observed that regardless of the number of available embryos, if there was at least one good grade embryo in the whole cohort, there was no benefit of transferring >2 embryos. If none of the embryos were of good grade, then transferring 3 embryos may increase the probability of pregnancy albeit at an insignificant level (Table 3.24, 3.25).

Hence, although previous guidelines limit the number of embryos transferred solely on the basis of the number of embryos available without accounting for embryo quality[90.91], it was shown that such guidelines can still inherently identify a sub-population who will not benefit from high-number ET; and we have further refined these population based protocols with the availability of embryological data. Literature supports the significance of this concurrent couple and embryo selection. It is shown that in a good prognostic group of women aged <34 years and in their first IVF treatment, strict embryo selection criteria are essential to maintain pregnancy rates[118]. The importance of embryo selection in elective single-ET is further emphasised by the drop in ongoing pregnancy rates from 36% to 13% when the embryo transferred is not of 'top grade'[516].

It was also observed that more 2-embryo transfers achieved ongoing pregnancy in the good-embryo grade dominant cycles when compared with the poor-embryo grade dominant cycles. However, in women who did not achieve pregnancy the percentage of 2- and 3-embryo transfers was the same in the good and poor prognostic groups. Hence, there are other factors that are not manifested by or operated through embryo quality, and increasing the number of embryos transferred cannot overcome the underlying pathology, so pregnancy is not achieved.

4.6 Multiple Pregnancy Following IVF Treatment

It has been demonstrated in previous sections that female age, duration of subfertility, and number of cleavage stage embryos available for transfer was predictive of the treatment outcome, but these variables interacted differently in the different combinations unique to individual women. For example, in younger women, longer durations of subfertility were more detrimental to pregnancy chances, and availability of a greater number of embryos for transfer could compensate for the decreased fertility chances of advanced female age. Each combination of these surrogate markers defines different levels of inherent fertility, reflecting the severity of the 'disease process' underlying the subfertility.

It is widely acknowledged that for optimum outcome, the intensity of treatment should be balanced with the severity of the disease; while less aggressive treatments may fail to overcome the pathology, more aggressive treatments may exert unacceptable side effects. A common practice in fertility treatment tries to achieve this balance, by titrating the number of embryos transferred against the perceived severity of the subfertility.

The main criticism of high-number ET is that it is an easy but risky way of overcoming fertility barriers. Embryo quality and endometrial receptivity are the key elements and their favourability can be estimated by clinical surrogate markers. The number of embryos to be transferred should be balanced with this estimation of favourability, rather than employing a blanket policy of multiple-embryo transfer, which inevitably increases the probability of multiple pregnancy. A study on elective single-ET concludes that the selection of a good prognostic group could keep an acceptable balance between reduction in multiple gestation and overall pregnancy rates[517].

The opponents of this policy argue that the sensitivity of the surrogate markers is low and so titration is not possible[122]. A common concern of those who argue against limiting the number of embryos transferred, is the iatrogenic lowering of pregnancy rates by the transfer of fewer embryos. They further argue that in the presence of poor quality embryos the number of embryos transferred needs to be increased to compensate for the fertility deficit.

We have already demonstrated that as women age and their duration of subfertility increases they require high-number ET to achieve pregnancy. However, women who produced a high number of embryos, despite their age and duration of subfertility, could achieve pregnancy without high-number ET, particularly if at least one of the embryos was of good grade.

The literature details similar approaches to optimise the number of embryos transferred. It is widely observed that the number of embryos transferred affects the probability of live birth, but that the implantation potential of the individual embryo approximated by the total number of embryos available for transfer, is more important[90]. Therefore, if a high number of embryos is available the probability of a live birth is greater than if only one or two embryos are available for transfer. When >4 embryos are available for transfer, the live birth rate is not affected by the transfer of two or three embryos, although there is a significant reduction in the multiple pregnancy rate when two embryos are transferred. This is shown to be valid for all female ages up to 40 years[90].

In the next section the prognostic factors of multiple pregnancy were evaluated from the perspective of the number of embryos transferred. In an analysis of 9700 ET procedures, the mean number of embryos implanted per transfer was 0.44 (range 0-4); 21% were singleton 9% were twin, and 1.7% were triplet.

Higher ovarian reserve, better ovarian response to stimulation and better embryo quality were associated with a higher probability of multiple pregnancy. Younger women with a shorter duration of subfertility were more likely to have a multiple pregnancy than singleton. Treatment cycles characterised by a greater number of 2-PN and cleavage stage embryos available for transfer were also at greater risk of multiple pregnancy, correlating with the quality of embryos transferred. Embryos with a lower grade of quality and higher stage of development were more likely to establish multiple pregnancy than singleton and the probability of multiple pregnancy was clearly associated with the number of embryos transferred (Table 3.30, 3.31).

A dose response relationship between these variables and the order of multiplicity (singleton vs twin vs high order) was observed but did not reach significance, with the exception of the number of embryos transferred, whereby both the probability of multiple pregnancy and the order of multiplicity increased with the increasing number of embryos transferred.

Both singleton and twin implantations were reduced in cycles complicated with ovarian cysts after down-regulation and in the presence of hydrosalpinx. The former probably acts by impeding the ovarian response, as shown in the previous section, and the latter by disturbing endometrial receptivity and embryo viability (Table 3.30, 3.31, *Table 3.82CD).*

Significant spontaneous fetal resorption following multiple implantation was noted. At delivery, only 59% of singleton pregnancies had one-embryo implantation, 34% two-embryo and 7% three-embryo. Likewise, 20% of twin pregnancies initially had triplet implantation, and 2% of triplet pregnancies had quadruplet implantation. Although the data were not available, it is unlikely that selective fetal reduction can explain the discrepancy between the number of embryos implanted and the number of babies born in singleton and twin pregnancies.

Logistic regression analysis was performed on 2400 cycles to predict singleton or multiple pregnancy. The number of embryos transferred was found to affect the multiplicity of pregnancy, even after correction for the effect of female age, the number of embryos available for transfer and the embryo quality. This differed from the prediction of pregnancy where the significance of the number of embryos transferred was lost after other clinical and embryological variables were corrected. Hence, the implantation potential of the embryo is linked to intrinsic factors unique to individual women but not to extrinsic factors such as the number of embryos transferred. However, the number of embryos implanted was linked with the number of embryos transferred (Table 3.32-3.34).

Schieve et. al.[94] conclude that the probability of multiple pregnancy from IVF treatment varies, not only with the number of embryos transferred but also with

female age. Women aged <35 years achieved maximum live-birth rates with as few as two embryos transferred. However, unlike the UK study[90], this retrospective cohort analysis indicates a statistically insignificant increase in live-birth rates among women aged >35 years if more than two embryos are transferred, but at the expense of a highly significant increase in multiple birth rates from 12% to 29%. If more than three embryos are transferred even in women with a poor prognosis (aged >36 years, previous unsuccessful IVF treatment and poor embryo quality), a significant trend toward higher multiple pregnancy rates remains[96].

It is obvious that factors that increase the probability of pregnancy also increase the probability of multiple pregnancy. This is not surprising because the underlying factor is the same: the implantation potential of each embryo and the receptivity of the endometrium. Factors associated with high embryo implantation rates lead to singleton pregnancy if one embryo is transferred, and to multiple pregnancy if more embryos are transferred.

These findings are in agreement with the published literature. The key determinants of success in IVF treatment, including female age, duration of subfertility and numbers of previously failed IVF attempts are also reported to affect the probability of multiple pregnancy[8,90]. It is this association that frames the current debate on minimising the multiple pregnancy probability without jeopardising the pregnancy chances. While transfer of a multiple number of embryos is proposed to compensate for the severity of subfertility, reflected by low implantation potential of the embryos or low endometrial receptivity, even to achieve singleton implantation, previous observations and publications indicate that this approach carries an unacceptably high probability of multiple pregnancy without necessarily increasing the pregnancy rates. Because what is being contemplated is not to correct the underlying pathology by improving embryo quality or endometrial receptivity, but simply to take advantage of the statistical probability of multiple independent events, no matter how low, by transferring multiple embryos in the hope that one will implant. If this is done indiscriminately there is a good possibility that some of the multiple ETs will be done in good prognosis patients with otherwise high implantation potential and

endometrial receptivity, with the inevitable outcome of high order multiple pregnancy.

From the earlier pioneering studies by Staessen[92,93,99] investigating the effect of quality and number of embryos transferred over the multiple pregnancy risks following IVF treatment, to the recent retrospective case control study by Licciardi et. al.[100] and HFEA-data analysis by Ozturk and Templeton[101], the same trend has persistently emerged; 'reducing the number of embryos transferred from three to two largely eliminates the occurrence of triplet pregnancies without altering the overall pregnancy rates'.

4.7 Prognostic Significance of Embryo Transfer Procedure

In IVF treatment, ET is the final and most crucial step. It is estimated that poor ET technique may account for as much as 30% of all failures in assisted reproduction[518]. There is little scientific evaluation of different ET protocols. However, it is customarily proposed that an easy, atraumatic transfer is important, with attention to factors such as patient preparation, medication, mock ET, choice of catheter, ultrasonographic guidance, and transfer technique.

In the current analysis, clinical variables defining different aspects of the ET procedure had no influence over the treatment outcome in terms of ongoing pregnancy rates. Despite the duration of ET being statistically shorter in women who achieved pregnancy, the difference was clinically negligible. Furthermore, there was no link between the duration of ET and its technical difficulty. The state of the catheter tip after the ET procedure, degree of discomfort experienced by the woman, degree of technical difficulty graded by the physician, and use of tenaculum, stylet or ultrasound guidance did not affect the ongoing pregnancy rates *(Table 3.39-3.44CD).*

These findings are in contrast with the published data. Blood or mucus, which may attach to the end of the catheter during ET, is shown to impede the embryo release. Blood on the outside of the catheter also has been related to difficult ET and poorer results[519]. It was shown that technically difficult ETs are

5 Low-dose Aspirin Co-treatment in Patients Undergoing IVF Treatment: Results

5.1 Patient Characteristics of the Aspirin and Placebo Groups

The study recruited 100 couples. Three couples randomized to the aspirin group later decided not to proceed with IVF treatment. From the remaining 97 couples, 46 women were in the aspirin group and 51 in the placebo group. All 97 couples completed their primary fresh IVF treatment cycle in accordance with the study protocol and routine policies of the Unit where the study was based. Patient characteristics, clinical and embryological details of the aspirin and placebo groups were given (Table 5.1-5.3). Details of female lifestyle in terms of smoking and alcohol consumption, and regularity of the menstrual cycle were detailed on the CD Rom *(Table 5.1-5.8CD)*. In the aspirin group 32% of the men presented with a sperm count <20 million/ml and 14.6% <5million/ml. In the placebo group, 16% of men had a sperm count <20 million/ml, and 10% <5 million/ml *(Table 5.9, 5.10CD)*.

Table 5.1: Subfertility in the Aspirin and Placebo Groups

	Subfertility	Female N (%)	Male N (%)	Couple N (%)
Aspirin	Primary	31(67.4 %)	30 (65.2%)	36 (78.3%)
	Secondary	15 (32.6%)	16 (34.8%)	10 (21.7%)
Placebo	Primary	30 (58.8%)	32 (62.7%)	37 (72.5%)
	Secondary	21 (41.2%)	19 (37.3%)	14 (27.5%)

N: Number of subjects

Table 5.2: Diagnosis of Subfertility in the Aspirin and Placebo Groups

Aetiology	Aspirin N (%)	Placebo N (%)
Unexplained	5 (10.9%)	13 (25.5%)
Male Factor	20 (43.5%)	13 (25.5%)
Ovarian	2 (4.3%)	2 (3.9%)
Endometriosis	4 (8.7%)	4 (7.8%)
Tubal Factor	15 (32.6%)	19 (37.3%)

Table 5.3: Patient Characteristics, Clinical and Embryological Variables of the Aspirin and Placebo Groups

		Aspirin Group		Placebo Group	
		N	Mean ± SD	N	Mean ± SD
Female Age (year)		46	32.4 ± 4.0	51	34.0 ± 4.4
Duration of Subfertility (month)		46	51.0 ± 38.2	51	53.9 ± 42.7
Gravity		46	0.7 ± 1.2	51	0.6 ± 1.0
Parity		46	0.2 ± 0.5	51	0.4 ± 0.9
BMI (kg/m^2)		46	24.0 ± 4.2	49	25.2 ± 5.7
Baseline FSH (IU/l)		46	6.7 ± 2.0	51	7.3 ± 3.9
Initial Gonadotrophin Dose (IU)		46	157 ± 31	51	157 ± 34
Duration of Gonadotrophin Stimulation (day)		46	12.4 ± 2.1	51	12.0 ± 2.3
Total Gonadotrophin Dose (IU)		46	2046 ± 728	51	1942 ± 725
E_2 Level after Down-Regulation (nmol/l)		46	0.10 ± 0.05	51	0.10 ± 0.07
Size of Baseline Ovarian Cyst (mm)		10	30.0 ± 9.0	11	24.9 ± 8.7
E_2 Level before hCG Injection (nmol/l)		46	5.3 ± 2.7	51	6.6 ± 4.4
Number of Follicles measuring	< 9 mm	46	2.8 ± 3.3	51	2.6 ± 4.6
	10-15 mm	46	6.0 ± 3.9	51	7.6 ± 5.9
	16-17 mm	46	2.5 ± 2.4	51	3.0 ± 2.1
	18-20 mm	46	1.7 ± 1.3	51	2.0 ± 1.4
	21-25 mm	46	0.8 ± 1.2	51	0.8 ± 1.4
	26-30 mm	46	0.23 ± 1.62	51	0.05 ± 0.24
Number of Follicles Aspirated		45	12.3 ± 6.4	49	12.4 ± 7.0
Number of Oocytes Collected		45	11.2 ± 6.5	49	11.2 ± 6.5
Number of Oocytes Used		44	10.7 ± 6.2	46	10.2 ± 5.4
Number of 2-PN Embryo		45	6.8 ± 4.6	49	6.6 ± 4.7
Number of Embryos Transferred		38	1.9 ± 0.2	43	1.9 ± 0.3
Number of Embryos Cryopreserved		45	3.7 ± 5.0	49	3.6 ± 4.7
Grade of the First Embryo		38	1.1 ± 0.5	43	1.2 ± 0.5
Grade of the Second Embryo		35	1.6 ± 0.8	40	1.5 ± 0.7
Grade of the Third Embryo		0		1	3.0
Embryo Transfer Time (sec)		38	31 ± 31	43	42 ± 59
Number of Embryos Implanted per Pregnancy in Fresh Cycle		14	1.35 ± 0.49	21	1.28 ± 0.46
Number of Embryos Implanted per Pregnancy in Fresh + FTER Cycle		19	1.31 ± 0.47	25	1.28 ± 0.45

N: Number of subjects whose data were available for each variable
BMI: Body mass index
Percentages represented as decimal of 1.0 ± SD

In the aspirin group 82% had Day 21 protocol and in the placebo groups 73%. The remaining used Day 2 protocol. Following down-regulation 21% of the women in both groups experienced ovarian cysts. ICSI was used in 41% of the aspirin group and 27% of the placebo group. Donor sperm was used only in a small subgroup of 4.3% in the aspirin group and 2% in the placebo group. Technical difficulty of the ET procedure was graded by the physician as easy in 83-84% of both groups, with only 5.3% in the aspirin group and 2.3% in the placebo group being graded difficult *(Table 5.11-5.15CD).*

In the aspirin group 82.6% had ET and in the placebo group 84.3%. In the aspirin group, 10.9% of couples elected cryopreservation of all embryos due to the high probability of ovarian hyper-stimulation syndrome (OHSS) and in the placebo group this was 9.8%. In the aspirin group 4.3% had failed fertilisation and in the placebo group 2%. Cycle cancellation before oocyte collection occurred in 2.2% of the aspirin group and 3.9% of the placebo group.

In the aspirin group the ongoing pregnancy rate was 23.9% and in the placebo group 33.3%. When cumulative pregnancy rates were calculated by combining the fresh IVF cycles and related FTER cycles, the ongoing pregnancy rates became 34.8% in the aspirin group and 39.2% in the placebo group (Table 5.4, 5.5). With low-dose aspirin co-treatment, the mean platelet volume increased from 8.6 to 9.2 during treatment for IVF. A similar increase from 8.8 to 9.1 was evident in the placebo group *(Table 5.16CD).*

Table 5.4: Treatment Outcome

	Ongoing Pregnancy after Fresh Treatments	**N (%)**
Aspirin	Ongoing Pregnancy	11 (23.9%)
	Miscarriage	3 (6.5%)
	Biochemical	2 (4.3%)
	Not Pregnant	22 (47.8%)
	No Embryo Transfer	8 (17.4%)
Placebo	Ongoing Pregnancy	17 (33.3%)
	Miscarriage	4 (7.8%)
	Biochemical	1 (2.0%)
	Not Pregnant	21 (41.2%)
	No Embryo Transfer	8 (15.7%)

Table 5.5: Cumulative Treatment Outcome

	Cumulative Ongoing Pregnancy after Fresh and Frozen Treatments	**N (%)**
Aspirin	Ongoing Pregnancy	16 (34.80%)
	Miscarriage	3 (6.50%)
	Biochemical	2 (4.30%)
	Not Pregnant	21(45.70%)
	No Embryo Transfer	4 (8.70%)
Placebo	Ongoing Pregnancy	20 (39.20%)
	Miscarriage	5 (9.80%)
	Biochemical	2 (3.90%)
	Not Pregnant	20 (39.20%)
	No Embryo Transfer	4 (7.80%)

5.1.1 Comparison of Patient Characteristics of the Aspirin and Placebo Groups

Differences in female age did not reach statistical significance in the aspirin and placebo groups (32 vs 34 years respectively). Duration of subfertility, previous obstetric history, regularity and duration of menstrual cycle, female height, weight, and BMI and female lifestyle in terms of smoking and alcohol consumption were similar in both groups. The distribution of primary and secondary female, male and couple subfertility and aetiologies of subfertility were similar in both groups. There was no difference in male weekly consumption of alcohol, sperm concentration, morphology, and motility grades between the aspirin and placebo groups *(Table 5.17-5.25CD).*

5.1.2 Comparison of Clinical and Embryological Variables of the Aspirin and Placebo Groups

When the clinical and embryological variables were compared, there were no differences between the aspirin and placebo groups for early follicular phase FSH values, type of the stimulation protocol (Day 21 vs Day 2), oestradiol levels after down-regulation and on the day of hCG administration, initial gonadotrophin dose, total gonadotrophin dose required for ovarian stimulation, duration of ovarian stimulation, follicular growth at different levels of maturation, total number of follicles aspirated, oocytes collected, and oocytes used for fertilisation, number of 2-PN embryos created, number of embryos cryopreserved and transferred, and the grade of the best two embryos selected for transfer.

The duration of the ET procedure, platelet count and mean platelet volume, both before and after ovarian stimulation, were similar in both groups *(Table 5.26CD)*.

IVF or ICSI and husband or donor sperm, were similarly employed in the aspirin and placebo groups. The occurrence of ovarian cysts following down-regulation was similar in both groups and the technical difficulty of ET procedures was graded similarly *(Table 5.27-5.33CD)*.

The distribution of cycles with ET and with no-ET due to elective embryo cryopreservation for OHSS, failed fertilisation, and cancellation before oocyte collection was similar. There was no difference between the two groups in ongoing pregnancy, biochemical pregnancy, miscarriage, and no-ET rates after the fresh IVF treatment study cycle and also for the cumulative rates of the fresh and the related FTER cycles (Table 5.6, 5.7). The number of embryos implanted per pregnancy was similar in the aspirin and placebo groups in the fresh cycle. The cumulative rate, including the related FTER cycle following the fresh cycle was also similar *(Table 5.26CD)*.

There was no difference between the aspirin and placebo groups for the mean platelet volume before and after ovarian stimulation. The mean platelet volume before and after ovarian stimulation were similar in the ongoing pregnancy, miscarriage, chemical pregnancy, no-pregnancy, and no-ET groups *(Table 5.34-5.36CD)*.

Table 5.6: Treatment Outcome in the Aspirin vs Placebo Groups

	Aspirin	**Placebo**	**p**
Ongoing Pregnancy	11	17	
Miscarriage	3	4	
Biochemical Pregnancy	2	1	NS
Not Pregnant	22	21	
No Embryo Transfer	8	8	

Table 5.7: Cumulative Treatment Outcome in the Aspirin vs Placebo Groups

Fresh + FTER	Aspirin	Placebo	p
Ongoing Pregnancy	16	20	
Miscarriage	3	5	
Biochemical Pregnancy	2	2	NS
Not Pregnant	21	20	
No Embryo Transfer	4	4	

5.2 Doppler Analysis

A total of 34 consecutive non-selected women aged 24-39 years in the same cohort of the aspirin study, were studied using colour-flow Doppler in 34 IVF treatment cycles. Elective cryopreservation of all embryos was performed in four cases because of the imminent risk of OHSS, and two IVF cycles were cancelled due to failed fertilisation. All of the women received GnRH agonist and recombinant FSH to achieve adequate ovarian stimulation. In 28 ET cycles, 13 clinical pregnancies were confirmed.

The patient and treatment cycle characteristics were summarized (Table 5.8-5.10). When women who conceived were compared with those who did not, no significant differences in any of the demographic parameters were observed except for the duration of subfertility. Longer delays in fertility were more common in women who did not conceive.

In the pregnant group, the day 10 serum oestradiol concentrations, number of developing follicles measuring 13 to 16mm, number of follicles aspirated, oocytes collected, and 2-PN embryos created were greater, and the duration of stimulation shorter with smaller doses of FSH stimulation.

Table 5.8: Patient Characteristics for Clinical Pregnancy

	Mean ± SD or Incidence		
	Pregnant	**Non-pregnant**	**P**
Age (year)	32.7 ± 4.8	33.8 ± 3.4	NS
Gravidity	0.9 ± 1.4	0.6 ± 0.7	NS
Parity	0.2 ± 0.4	0.4 ± 0.7	NS
BMI (kg/m^2)	25 ± 6.1	25 ± 4.3	NS
Duration of subfertility (year)	3.5 ± 1.6	5.9 ± 3.8	<0.05
Type of the subfertility (couple)			
Primary	7 (53%)	11 (73%)	NS
Secondary	6 (47%)	4 (27%)	NS
Parity			
Parous	3 (23%)	4 (26%)	NS
Nulliparous	10 (77%)	11 (73%)	NS

Table 5.9: Diagnosis of Subfertility for Clinical Pregnancy

Pregnancy	**Aetiology**				**p**
	Male	**Tubal**	**Unexplained**	**Ovarian**	
Yes	6	7	0	0	
No	5	5	3	2	NS
Total	11	12	3	2	

Table 5.10: IVF Cycle Characteristics for Clinical Pregnancy

	Mean ± SD		
	Pregnant	**Non-pregnant**	**p**
Number of days of stimulation	11.5 ± 1.7	13.6 ± 2.5	<0.05
Total dose of FSH used (IU)	1741 ± 331	2398 ± 827	<0.05
Day 10 E_2 (nmol/l)	2.7 ± 1.9	1.4 ± 1.2	<0.05
Number of Day 10 follicles (13-16 mm)	5.6 ± 4.6	2 ± 1.5	<0.05
Number of follicles aspirated	11.3 ± 4.7	7.4 ± 4.2	<0.05
Number of oocytes collected	10.1 ± 4.5	6.4 ± 3.9	<0.05
Number of 2-PN embryos	5.8 ± 2.9	3.6 ± 2.7	<0.05

There were no statistically significant differences in Doppler indices (PI, RI, Vmax, Vmin) for right and left uterine artery impedance and so the mean value for each woman was calculated and used for subsequent comparison *(Table 5.39, 5.40CD).*

Throughout the different stages of the IVF treatment (baseline and the last day of ovarian stimulation), there was a strong linear correlation among the different Doppler indices of PI, RI, S/D for uterine, sub-endometrial and follicular blood flows *(Table 5.41-5.44CD).*

In the pregnant and non-pregnant groups, there was no difference in mean baseline uterine artery impedance (PI and RI), but in the pregnant group the last day flow before hCG injection in the ascending uterine arteries was characterised by significantly lower PI values (Table 5.11).

During ovarian stimulation, when compared with baseline values there was a significant decrease in the PI values on the day of the last ultrasound scan before hCG injection in the pregnant group. This drop in vascular resistance was not seen in the non-pregnant group (Table 5.11).

Table 5.11: Uterine Artery Doppler Indices for Clinical Pregnancy

	Mean ± SD		
	Pregnant	**Non-pregnant**	**p**
Baseline uterine artery PI	2.9 ± 1.3	2.4 ± 0.9	NS
Baseline uterine artery RI	0.8 ± 0.1	0.8 ± 0.0	NS
Last day uterine artery PI	2.0 ± 0.5	2.8 ± 1.3	<0.05
Last day uterine artery RI	0.7 ± 0.0	0.8 ± 0.1	0.08
Baseline – Last day uterine artery PI	0.9 ± 1.4	-0.2 ± 0.4	<0.05

When measured by S/D ratio rather than PI and RI, lower uterine artery impedance lost its significant association with the treatment outcome, because of the undefined S/D values in cases of absent end diastolic blood flow *(Table 5.45CD)*.

In the pregnant group, the last day peri-follicular blood flow was characterised by lower vascular impedance but this did not reach statistical significance. The follicle with the most extensive perifollicular vascular perfusion on colour mapping was selected for assessment (Table 5.12).

Table 5.12: Peri-follicular Blood Flow to the Dominant Follicle for Clinical Pregnancy

	Mean ± SD		
	Pregnant	**Non-pregnant**	**p**
Last day follicle PI	0.7 ± 0.1	1.1 ± 0.6	NS
Last day follicle RI	0.5 ± 0.0	0.5 ± 0.1	NS
Last day follicle S/D	2.1 ± 0.3	2.5 ± 1.2	NS
Last day peri-follicular vascularity	24% ± 8%	24% ± 13%	NS

In the pregnant and non-pregnant groups, there was no significant difference in mean sub-endometrial vascular impedance during ovarian stimulation, with the exception of baseline sub-endometrial RI values, which were slightly higher in the pregnant group (Table 5.13).

Table 5.13: Sub-Endometrial Blood Flow for Clinical Pregnancy

	Mean ± SD		
	Pregnant	**Non-pregnant**	**p**
Baseline Sub-endometrial PI	1.8 ± 0.9	1.6 ± 1.4	NS
Baseline Sub-endometrial RI	0.7 ± 0.1	0.5 ± 0.1	<0.05
Baseline Sub-endometrial S/D	3.9 ± 3.0	3.6 ± 13.9	NS
Last day Sub-endometrial PI	1.3 ± 0.7	1.3 ± 1.0	NS
Last day Sub-endometrial RI	0.6 ± 0.2	0.6 ± 0.1	NS
Last day Sub-endometrial S/D	2.5 ± 0.9	2.9 ± 1.7	NS

No significant association was detected between the uterine artery vascular impedance values and corresponding oestradiol levels, baseline FSH values and BMI (Table 5.14).

Table 5.14: Correlations between Uterine Artery PI and Clinical Variables

			E_2		
Uterine Artery		**BMI**	**Baseline**	**Last day**	**FSH**
Baseline PI	Correlation Coefficient	0.12	-0.03	0.17	-0.13
	p	NS	NS	NS	NS
Last day PI	Correlation Coefficient	0.20	0.20	0.009	-0.25
	p	NS	NS	NS	NS

During ovarian stimulation endometrial growth was comparable in the pregnant and non-pregnant groups and there were no significant differences in endometrial thickness at the end of ovarian stimulation. Further, there was no linear correlation of endometrial thickness with E_2 levels, Doppler indices of the uterine artery, or sub-endometrial blood flow at baseline and the day of the last ultrasound scan (Table 5.15, 5.16).

Table 5.15: Endometrial Thickness for Clinical Pregnancy

	Mean ± SD		
	Pregnant	**Non-pregnant**	**p**
Baseline Endometrial Thickness	2.4 ± 1.7 mm	2.3 ± 0.9 mm	NS
Last day Endometrial Thickness	11.4 ± 2.7 mm	10.7 ± 2.8 mm	NS

Table 5.16: Correlations between Endometrial Thickness and (E_2 level, Uterine Artery PI, Sub-endometrial PI)

	Correlation Coefficient (p)		
	E_2 level	**Uterine artery PI**	**Sub-endometrial PI**
Baseline Endometrial Thickness	-0.16 (NS)	-0.20 (NS)	-0.01 (NS)
Last day Endometrial Thickness	0.21 (NS)	0.10 (NS)	-0.10 (NS)

In the pregnant group the last day uterine artery resistance was significantly lower. An ROC curve was plotted to identify the optimum cut-off point for the last day uterine artery PI, which separated the good and poor prognostic groups. The aim was to locate a PI cut-off level above which none of the women in the poor prognostic group would achieve pregnancy, so that the poorest response group could be identified (sensitivity 0.00). When the cut-off level was 3.00 (Area: 0.31, SE: 0.10, 95%CI: 0.1 to 0.5) ROC curve analysis revealed the test sensitivity to be 0.00 with specificity 0.54. Therefore, in women with PI values >3.00, the probability of pregnancy is very low when predicted by the last day uterine artery Doppler. This was further tested by categorising the study population into two groups based on their PI values being ≤ or > 3 (Table 5.17).

Table 5.17: Last Day Uterine Artery PI for Clinical Pregnancy

Pregnancy	**Last Day Mean Uterine Artery PI**		**p**
	≤ 3	**> 3**	
Yes	13	0	<0.01
No	8	7	

There was no difference in the baseline uterine artery vascular resistance between the pregnant and non-pregnant groups. The hypothetical PI cut-off level of 14.5 minimised the sensitivity of the test to 0.00 (Area: 0.52, SE: 0.11, 95% CI: 0.3 to 0.7). Therefore, at the baseline investigation, the conventional PI cut-off level of 3 did not prognostically identify the women (Table 5.18).

Table 5.18: Baseline Uterine Artery PI for Clinical Pregnancy

Pregnancy	Baseline Mean Uterine Artery PI		p
	≤ 3	>3	
Yes	7	6	NS
No	8	7	

The last day peri-follicular vascular resistance of the dominant follicle was lower in women in the pregnant group, however the difference in mean values did not reach a level of statistical significance. An ROC curve was plotted to identify the optimum cut-off point for statistical significance (Area: 0.37, SE: 0.12, 95% CI: 0.1 to 0.6). In determining the prognosis, when the cut-off level was 1.08, the sensitivity of the test was 0.00 with a specificity of 0.73. Therefore, when predicted by peri-follicular blood flow, with PI values >1 the probability of pregnancy was very low. This was further tested by categorising the study population based on PI values < or >1, and the probability of pregnancy was significantly different between the two groups (Table 5.19).

Table 5.19: Last Day Follicular PI for Clinical Pregnancy

Pregnancy	Last day Follicular PI		p
	≤ 1	>1	
Yes	11	0	<0.05
No	8	3	

When the same approach was applied to sub-endometrial vascular resistance values at baseline and the day of the last scan, very high cut-off levels beyond the maximum observed test values were required to achieve 0.00 sensitivity. Therefore, there were no practical cut-off levels at which the statistical significance could be tested for good or poor prognosis (Table 5.20).

Table 5.20: Baseline and Last Day Sub-endometrial PI for Clinical Pregnancy

Pregnancy	Baseline Sub-endometrial PI		p
	≤ 3	>3	
Yes	8	5	NS
No	7	8	
	Last Day Sub-endometrial PI		
Yes	11	2	NS
No	11	4	

Women with a last day uterine artery PI value >3, also had poorer perifollicular vascularisation and sub-endometrial perfusion, and fewer maturing follicles and collected oocytes. Higher alcohol consumption appeared to be associated with lower uterine artery resistance (Table 5.21).

Table 5.21: Variables for Last Day Uterine Artery Doppler Indices with 'cut off = 3'

	Mean ± SD		
	High Resistance	Low Resistance	p
Number of follicles (8-12mm) on the last scan day	2.4 ± 1.9	4.7 ± 4.8	<0.05
Number of follicles (13-16mm) on the last scan day	2.8 ± 2.2	5.5 ± 4.4	<0.05
Number of aspirated oocytes	7.5 ± 4.1	11 ± 7.1	0.08
Peri-follicular vascularity on the last scan day (%)	0.1 ± 0.0	0.2 ± 0.1	0.01
Peri-follicular PI on the last scan day	1.3 ± 0.8	0.7 ± 0.1	<0.01
Sub-endometrial PI on the last scan day	1.7 ± 1.2	1.1 ± 0.5	0.07
Female alcohol intake (unit / week)	1.7 ± 2.3	8.0 ± 10.4	<0.01

High resistance: Vascular resistance above the cut off Low resistance: Vascular resistance below the cut off

At the baseline scan there was a significant linear correlation between female smoking and higher sub-endometrial vascular resistance (Table 5.22).

Table 5.22: Female Smoking for Baseline Sub-endometrial PI, RI

		Baseline Sub-endometrial PI	Baseline Sub-endometrial RI
Female smoking	Pearson Correlation	0.39	0.38
	p	<0.05	<0.05

With the last day follicular PI cut-off level of 1.5 the sensitivity of the test was minimised to 0.00, as with the cut-off level 1, but with a higher specificity of 80%, thereby ensuring that the women with the lowest probability of pregnancy could be identified with less probability of including those with a better prognosis (Area: 0.37, SE: 0.12, 95% CI: 0.1 to 0.6)

When this cut-off level was applied to identify the poorest prognostic group, women with a PI value of >1.5 had lower oestrogen concentrations, fewer mature follicles, fewer follicles aspirated, fewer oocytes collected, and fewer embryos created. This suggested an association between poor follicular perfusion, poor ovarian response, and poor clinical outcome (Table 5.23).

Table 5.23: Variables for the Last Day Peri-follicular Doppler Indices with 'cut off = 1.5'

	Mean ± SD		
	High Resistance	**Low Resistance**	**p**
Last scan day E_2 concentration (nmol/l)	1.3 ± 0.0	4.9 ± 2.8	<0.01
Number of follicles (8-12mm) on the last scan day	2.0 ± 0.0	4.5 ± 4.8	<0.01
Number of follicles (>20mm) on the last scan day	0.0 ± 0.0	1.1 ± 1.1	<0.01
Number of follicles aspirated	4.5 ± 0.7	12.3 ± 7.0	<0.01
Number of oocytes collected	3.5 ± 2.1	11.0 ± 7.0	<0.05
Number of 2-PN embryos	1.0 ± 0.0	6.4 ± 4.8	<0.01
Number of embryos cryopreserved	0.0 ± 0.0	3.4 ± 5.1	<0.01

High resistance: Vascular resistance above the cut off Low resistance: Vascular resistance below the cut off .

When compared with the placebo group, co-treatment with aspirin 150 mg per day did not improve any of the Doppler Indices of the uterine, sub-endometrial, and follicular blood flow or the follicular vascularity and endometrial thickness at any significant level either soon after pituitary down-regulation or during the ovarian stimulation *(Table 5.46CD).*

5.3 VEGF-VEGFR Concentrations in the Aspirin and Placebo Groups

Serum VEGF and VEGFR levels were measured in 80 women during pre-treatment natural cycles, and in 59 of these on the day of oocyte collection. Four samples of follicular fluid were discarded due to blood contamination. Follicular fluid levels were measured in 55 women from the same cohort (Table 5.24). In the aspirin and placebo groups there was no significant difference in pre- and post-treatment serum levels of VEGF (pg/ml) and VEGFR (pg/ml), or follicular fluid concentrations (Table 5.25).

Table 5.24: VEGF and VEGFR Concentrations

	Pre-Treatment Serum VEGF	Post-Treatment Serum VEGF	Follicular Fluid VEGF	Pre-Treatment Serum VEGFR	Post-Treatment Serum VEGFR	Follicular Fluid VEGFR
N	80	59	55	80	59	55
Mean ± SD	285.0 ± 203.3	297.1 ± 179.2	3107.5 ± 1636.3	32.8 ± 12.3	35.0 ± 10.6	4470.7 ± 1755.7

VEGF and VEGFR Concentrations: pg/ml.

Table 5.25: VEGF and VEGFR Concentrations for the Aspirin vs Placebo Groups

		N	Mean ± SD	p
Pre-treatment Serum VEGF	Aspirin	36	265.2 ± 176.6	NS
	Placebo	43	299.4 ± 225.8	
Post-treatment Serum VEGF	Aspirin	29	288.3 ± 169.8	NS
	Placebo	30	305.6 ± 190.3	
Follicular Fluid VEGF	Aspirin	27	2738.7 ± 1318.0	NS
	Placebo	28	3463.2 ± 1848.0	
Pre-treatment Serum VEGFR	Aspirin	36	34.1 ± 12.6	NS
	Placebo	43	31.5 ± 12.3	
Post-treatment Serum VEGFR	Aspirin	29	35.0 ± 7.7	NS
	Placebo	30	35.1 ± 13.0	
Follicular Fluid VEGFR	Aspirin	27	4062.8 ± 1736.1	NS
	Placebo	28	4864.2 ± 1713.4	

VEGF and VEGFR Concentrations: pg/ml.

5.3.1 VEGF-VEGFR Concentrations in Different Outcome Groups

In the ET and no-ET groups there was no difference in the pre- and post-treatment serum VEGF and VEGFR levels, and the FF VEGF levels were similar. However, FF VEGFR levels were significantly lower in the no-ET group (Table 5.26, *Table 5.47CD).*

Table 5.26: Comparison of VEGFR levels for ET and No-ET Groups

Embryo Transfer	N	Follicular Fluid VEGFR (Mean ± SD)	p
Yes	50	4635.5 ± 1744.7	0.01
No	5	2823.1 ± 770.8	

VEGFR Concentrations: pg/ml.

There were no differences in pre- and post-treatment serum VEGF and VEGFR or FF VEGF levels in the ET, no-ET due to elective cryopreservation of all embryos to prevent OHSS, no ET due to failed fertilisation, and no-ET due to cancellation before oocyte collection groups. However, FF VEGFR concentrations were lower in the no-ET group due to elective cryopreservation of all embryos than in the ET group (3038 vs 4635 pg/ml; p:<0.05) *(Table 5.48CD).*

In the pregnant, non-pregnant and no-ET groups there was no difference in the levels of pre- and post-treatment serum VEGF and VEGFR. The levels of FF VEGF were also similar, but the levels of FF VEGFR were significantly lower (Table 5.27, *Table 5.49CD).*

Table 5.27: Comparison of Follicular Fluid VEGFR for Clinical Pregnancy

Pregnant	N	Follicular Fluid VEGFR (Mean ± SD)	p
Yes	26	4529.1 ± 1800.5	
No	24	4750.8 ± 1713.0	<0.05
No ET	5	2823.1 ± 770.8	

VEGFR Concentrations: pg/ml.

In the ongoing pregnancy, no-ongoing pregnancy and no-ET groups, there was no difference in the levels of pre- and post- treatment serum VEGF and VEGFR. The levels of follicular fluid VEGF were similar, but there were significant differences in the levels of FF VEGFR (Table 5.28, *Table 5.50CD).*

Table 5.28: Comparison of Follicular Fluid VEGFR for Ongoing Pregnancy

Ongoing Pregnancy	N	Follicular Fluid VEGFR (Mean ± SD)	p
Yes	20	4525.6 ± 1924.8	
No	30	4708.8 ± 1643.7	<0.05
No ET	5	2823.1 ± 770.8	

VEGFR Concentrations: pg/ml.

In the ongoing pregnancy, miscarriage, biochemical pregnancy, no-pregnancy and no-ET groups, there was no difference in the levels of pre- and post-treatment serum VEGF and VEGFR, and the levels of FF VEGF and VEGFR were similar *(Table 5.51CD).* In the fresh IVF treatment study cycle and related FTER cycle, the cumulative pregnancy rates were calculated and the data evaluated for the ongoing pregnancy, biochemical pregnancy, miscarriage, no-pregnancy and no-ET groups. There was no difference in the levels of pre-and post-treatment serum VEGF and VEGFR or FF VEGF and VEGFR *(Table 5.52CD).*

5.3.2 VEGF-VEGFR Concentrations and Number of Embryos Implanted

In the fresh IVF treatment cycles, the number of implanted embryos did not correlate with the levels of pre- or post-treatment serum VEGF and VEGFR or FF VEGF and VEGFR. Although not reaching a level of statistical significance, there was a consistent negative association between implantation and the levels of pre- and post-treatment serum VEGFR and FF VEGF and VEGFR. However, when the cumulative implantation rate of the fresh and related FTER cycles was evaluated, the number of implanted embryos negatively correlated with the levels of FF VEGF (p: 0.01).

Pre-treatment levels of serum VEGF correlated positively with post-treatment levels of serum VEGF (p: <0.001), but negatively with pre-treatment serum VEGFR (p: <0.01) and post-treatment serum VEGFR (p: <0.05). However, there was no correlation with FF VEGF or its receptor. The levels of post-treatment serum VEGF correlated negatively with pre- (p: <0.01) and post- (p: <0.05) treatment serum VEGFR. There was no correlation with FF VEGF or its receptor. The levels of FF VEGF correlated positively with the levels of its receptor in follicular fluid (p: <0.05) and negatively with pre-treatment serum

VEGFR (p: <0.05). Pre-treatment serum VEGFR correlated positively with post-treatment serum VEGFR (p: 0.001) *(*Figure 5.1, 5.2, *Table 5.53-5.56CD).*

Figure 5.1: Correlations between VEGF-VEGFR Concentrations (1)

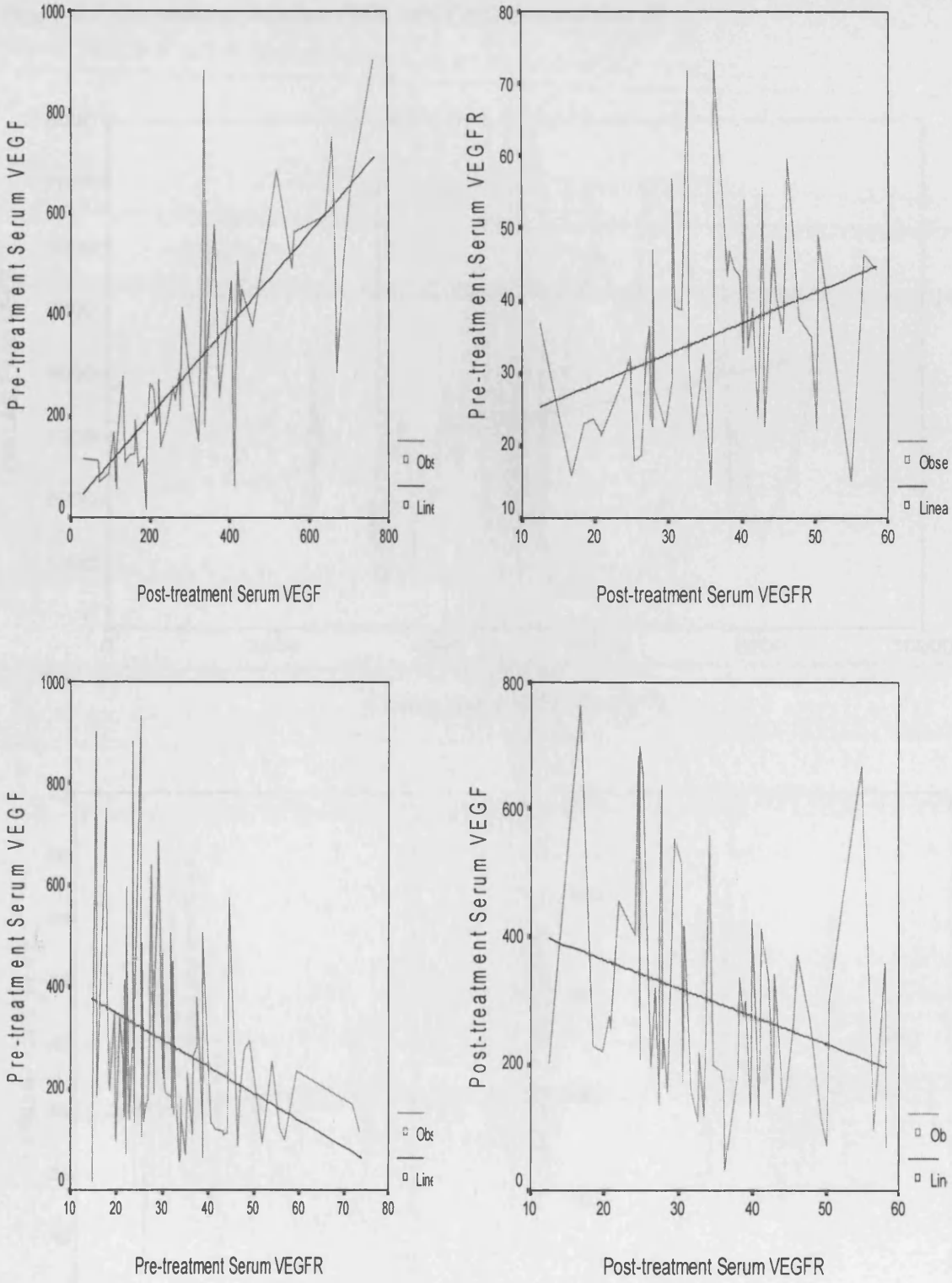
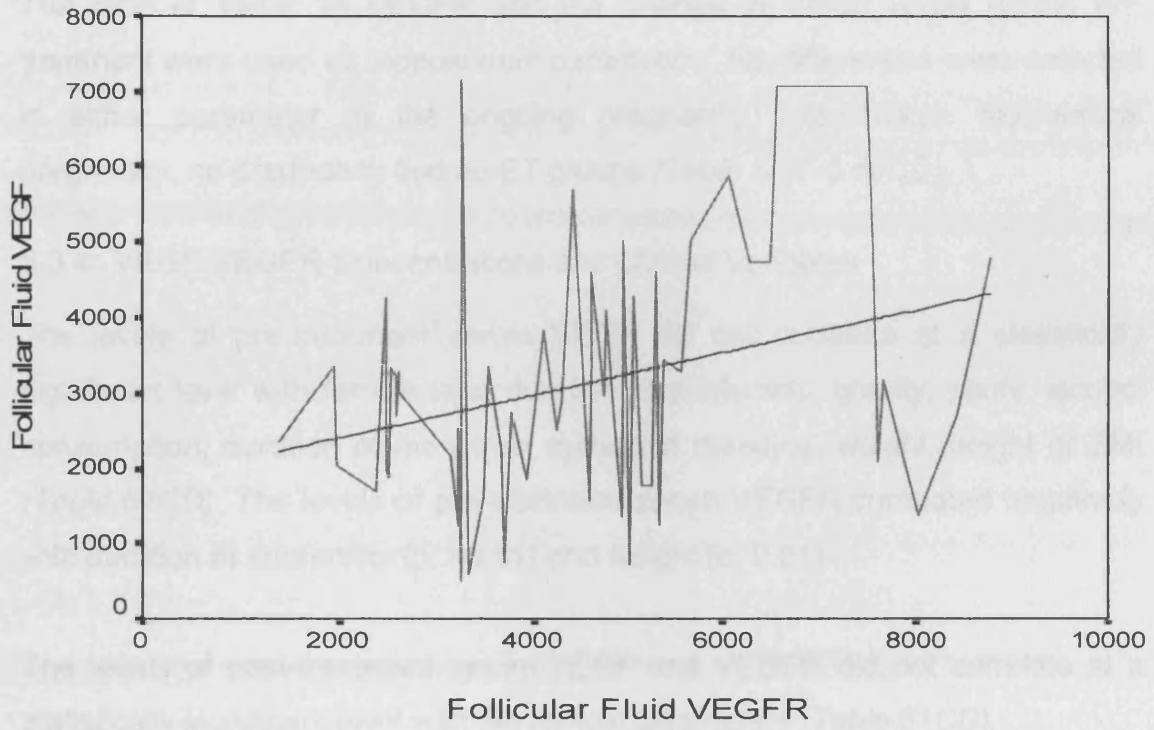
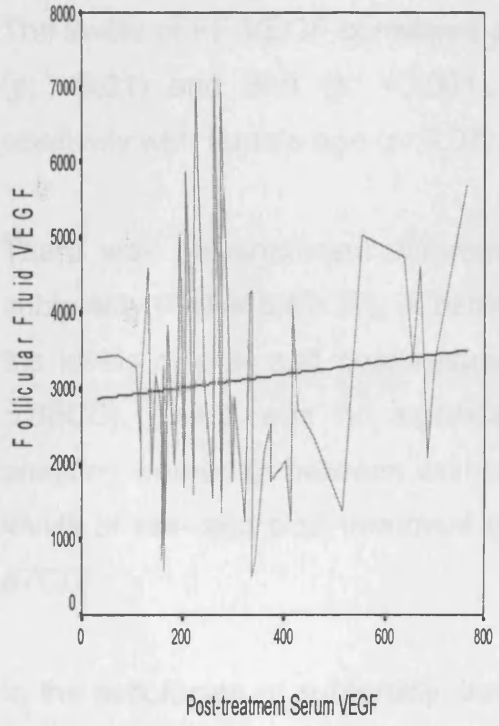
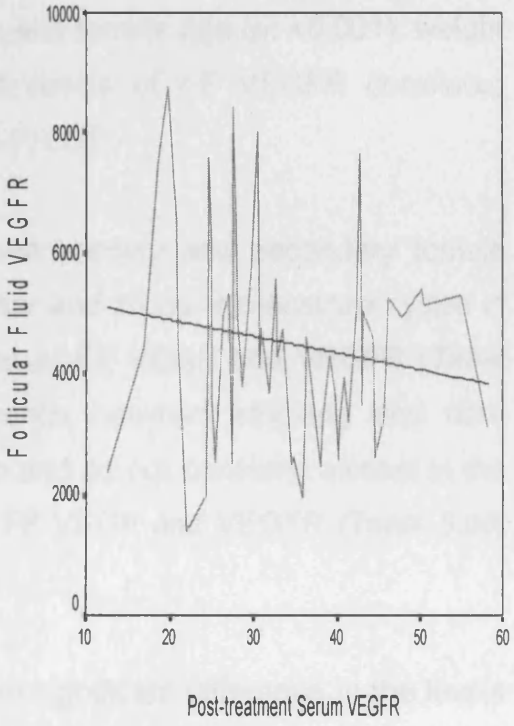


Figure 5.2: Correlations between VEGF-VEGFR Concentrations (2)







5.3.3 VEGF:VEGFR Ratio and Difference Between Pre- and Post-treatment VEGF Concentrations

The ratio of VEGF to VEGFR and the change in VEGF levels during IVF treatment were used as independent parameters. No differences were detected in either parameter in the ongoing pregnancy, miscarriage, biochemical pregnancy, no-pregnancy and no-ET groups *(Table 5.57-5.60CD).*

5.3.4 VEGF-VEGFR Concentrations and Clinical Variables

The levels of pre-treatment serum VEGF did not correlate at a statistically significant level with female age, duration of subfertility, gravity, parity, alcohol consumption, duration of menstrual cycle and bleeding, weight, height or BMI *(Table 61CD).* The levels of pre-treatment serum VEGFR correlated negatively with duration of subfertility (p: <0.01) and weight (p: 0.01).

The levels of post-treatment serum VEGF and VEGFR did not correlate at a statistically significant level with the clinical parameters *(Table 61CD).*

The levels of FF VEGF correlated positively with female age (p: <0.001), weight (p: <0.01) and BMI (p: <0.001), and the levels of FF VEGFR correlated positively with female age (p: 0.01) *(Table 5.61CD).*

There was no significant difference between primary and secondary female subfertility (*Table 5.62CD*), or between regular and irregular menstrual cycles in the levels of pre- and post-treatment serum or FF VEGF and VEGFR (*Table 5.65CD*). There was no significant difference between smoking and non-smoking women or between women who do and do not consume alcohol in the levels of pre- and post-treatment serum or FF VEGF and VEGFR *(Table 5.66, 67CD).*

In the aetiologies of subfertility, there was no significant difference in the levels of pre- and post-treatment serum or FF VEGF and VEGFR. However, the absolute values for VEGF in serum and follicular fluid showed a trend of endometriosis > unexplained > tubal factor = male factor > ovarian factor. The

absolute values for VEGFR in serum and follicular fluid showed a trend of male factor > tubal factor > unexplained > endometriosis > ovarian *(Table 5.68CD).*

5.3.5 VEGF-VEGFR Concentrations and IVF Variables

Patient characteristics, clinical variables of ovarian stimulation and IVF that correlated with the VEGF and VEGFR concentrations were summarized (Table 5.29, 5.30). Positive correlation between the variables was given as '↑', negative correlation as '↓', and no correlation as "↔". Correlations with a p value of <0.05 were marked as '*', and those with a p value of 0.05-0.09 as '**'. Numerical values of the correlation coefficients and corresponding p values were given on the CD Rom (*Table 5.69CD).*

There was no difference in the levels of pre- and post-treatment serum VEGF and VEGFR or FF VEGF and VEGFR between women who developed ovarian cysts following down-regulation and those who did not *(Table 5.70CD).*

Table 5.29: Correlations between Clinical Variables of Ovarian Stimulation, IVF and the VEGF, VEGFR Concentrations

		Pre-treatment Serum		Post-treatment Serum		Follicular Fluid	
		VEGF	VEGFR	VEGF	VEGFR	VEGF	VEGFR
Baseline FSH		↓*	↔	↔	↔	↔	↑**
Duration of Gonadotrophin Stimulation		↔	↔	↔	↔	↑*	↑*
Total Gonadotrophin Dose		↔	↔	↔	↔	↑*	↑*
Oestrogen Level after Down-regulation		↔	↔	↑*	↔	↔	↔
Oestrogen Level before hCG Injection		↔	↔	↔	↔	↓*	↔
Number of Follicles	< 9 mm	↔	↔	↓**	↔	↔	↔
	10-15	↔	↔	↔	↔	↓*	↓*
	16-17	↔	↔	↔	↔	↓*	↔
	18-20	↔	↔	↔	↔	↔	↔
	21-25	↔	↔	↔	↔	↔	↔
	26-30	↔	↔	↔	↔	↔	↔
Follicles Aspirated		↔	↔	↔	↔	↓*	↓**
Oocytes Collected		↔	↔	↔	↔	↓*	↓*
Oocytes Used		↔	↔	↔	↔	↓*	↓*
2-PN Embryo		↓**	↔	↔	↔	↓*	↔
Number of Embryos Transferred		↔	↔	↔	↔	↔	↔
Number of Embryos Cryopreserved		↓*	↔	↓**	↔	↓*	↔
Grade of the First Transferred Embryo		↔	↔	↔	↔	↔	↔
Grade of the Second Transferred Embryo		↔	↔	↔	↔	↔	↔
Mean Platelet Volume Before Treatment		↔	↔	↔	↔	↔	↔
Mean Platelet Volume After Treatment		↔	↔	↔	↔	↔	↔

↑: Positive correlation between the variables, ↓: negative correlation, ↔ : no correlation, * : p:<0.05, ** : p:0.05-0.09.

Table 5. 30: Summary of the Significant Correlations between Clinical Variables of Ovarian Stimulation, IVF, Treatment Outcome and the VEGF, VEGFR Concentrations

		Pregnant vs Not-pregnant vs no-ET	ET vs no-ET	Positive Correlation	Negative Correlation
Pre-treatment Serum	**VEGF**	NS	NS		FSH Number of Embryos cryo-preserved
	VEGFR	NS	NS		Subfertility duration Weight
Post-treatment Serum	**VEGF**	NS	NS	E_2 after down- regulation	
	VEGFR	NS	NS		
Follicular Fluid	**VEGF**	↓ in pregnant (NS)	↓ in no-ET (NS)	Age Weight, BMI Stimulation duration Total FSH dose	Implantation in Fresh + FTER E_2 before hCG Number of 10-17mm follicles Follicles aspirated Oocytes collected Embryos created Embryos cryo-preserved
	VEGFR	↓ in no-ET due to imminent hyperstimulation risk		Age Stimulation duration Total FSH dose	Number of 10-15 mm follicles Oocytes collected

NS: Not significant. Associations not reaching the level of significance are marked as NS.

5.3.6 VEGF-VEGFR Concentrations and Doppler Variables

Correlations between sub-endometrial and the uterine artery Doppler variables and VEGF, VEGFR concentrations during ovarian stimulation were summarized (Table 5.31). Positive correlation between the variables was given as '↑', negative correlation as '↓'. Correlations with a p value of <0.05 were marked with '*', and those with a p value of 0.05-0.09 with '**'. Poor perfusion was defined by the combination of a high PI ± low drop in PI during the treatment. In this assessment, correlations with the PI had the priority to define the perfusion status unless the correlations with the level of drop in PI had a statistically higher significance. Correlations with a p value of >0.09 were included only if the direction of their associations with the VEGF-VEGFR concentrations persistently fitted a common theme of poor or good perfusion. Numerical values of the correlation coefficients and corresponding p values were given in the CD Rom *(Table 5.71, 5.72CD).*

Table 5.31: Correlations between Doppler Variables and the VEGF, VEGFR Concentrations

		Pre-treatment Serum		Post-treatment Serum		Follicular Fluid	
		VEGF	**VEGFR**	**VEGF**	**VEGFR**	**VEGF**	**VEGFR**
Uterine Artery Perfusion	Baseline PI	↑**	↓	↑*	↓	↑	↑
	Last Day PI	↑	↓	↑		↑	↑
	Last Day – Baseline PI	↓*	↑	↓**	↑*		
Poor Uterine Artery Perfusion		**High VEGF**	**Low VEGFR**	**High VEGF**	**Low VEGFR**	**High VEGF**	**High VEGFR**
Sub-endometrial Perfusion	Baseline PI	↑	↓	↑	↓		↑
	Last Day PI	↑	↓*	↑	↓	↑**	↑
	Last Day – Baseline PI	↓			↑		↓
Poor Sub-endometrial Perfusion		**High VEGF**	**Low VEGFR**	**High VEGF**	**Low VEGFR**	**High VEGF**	**High VEGFR**

↑: Positive correlation between the variables, ↓: negative correlation, * : p:<0.05, ** : p:0.05-0.09.
Uterine artery perfusion: Positive correlation of PI values and VEGF concentration (High resistance associated with high VEGF), and negative correlation of the drop in PI values during treatment and VEGF concentration (Lower levels of drop in PI values during treatment associated with high VEGF). Because high PI values and a low drop in PI values during the treatment referred to poor perfusion, high VEGF concentrations were associated with poor uterine artery perfusion.

5.3.7 Multivariate Analysis of Doppler Variables of Utero-ovarian Blood Flow and VEGF and VEGFR for Prediction of Pregnancy

Logistic regression analysis on 53 subjects revealed the late follicular phase-uterine artery Doppler indices during ovarian stimulation and post-treatment serum VEGFR levels as the independent prognosticators. The following independent variables were tested for prognostic significance using the forward stepwise conditional entry method (Table 5.32, 5.33).

Table 5.32: Independent variables used in the Logistic Regression Analysis (1)

Female Age
Duration of Subfertility
Parity
Gravity
BMI
Sperm Parameters
Aetiology
Protocol
Total Gonadotrophin Dose
Duration of Gonadotrophin Stimulation
Baseline Oestrogen Level
Day 10 Oestrogen Level
Last Day Oestrogen Level
Number of Follicles Aspirated
Number of Oocytes Aspirated
Number of Embryos Created
Grade of the First Embryo
Grade of the Second Embryo
Cryopreserved Embryos
Type of IVF
Treatment Group
Pre Treatment Serum VEGF
Post Treatment Serum VEGF
Follicular Fluid VEGF
Pre Treatment Serum VEGFR
Post Treatment Serum VEGFR
Follicular Fluid VEGF Receptor
Mean Platelet Volume Before Treatment
Mean Platelet Volume After Treatment
Differences in Platelet Count
Difference in Mean Platelet Volume
Baseline Endometrial Thickness
Last Day Endometrial Thickness
Uterine Artery Doppler Values
Sub-endometrial Doppler Values
Peri-follicular Doppler Values

Table 5.33: Variables in the Equation for Clinical Pregnancy (1)

	Regression Coefficient	**SE**	**OR**	**p**	**R^2**
Uterine Artery PI	8.84	3.81	>10.00	<0.05	0.34
Post Treatment Serum VEGFR	0.10	0.04	1.10	<0.05	
Constant	-9.96	3.62	0.00	0.01	

A more focused logistic regression analysis was performed on 35 patients using the forced entry method. The dependent variable was pregnancy, and independent variables were assessed for their prognostic significance (Table 5.34, 5.35).

Table 5.34: Independent Variables used in the Logistic Regression Analysis

Pre Treatment Serum VEGF
Post Treatment Serum VEGF
Follicular Fluid VEGF
Pre Treatment Serum VEGFR
Post Treatment Serum VEGFR
Follicular Fluid VEGFR
Uterine Artery Doppler Values
Peri-follicular Doppler Values

Table 5.35: Variables in the Equation for Clinical Pregnancy (2)

	Regression Coefficient	**SE**	**OR**	**p**
Pre Treatment Serum VEGF	0.02	0.01	1.02	0.05
Post Treatment Serum VEGF	-0.02	0.01	0.98	0.05

Only pre- and post- treatment serum VEGF concentrations were significant and none of the Doppler indices maintained their univariate discriminative power. This may further indicate the primacy of VEGF control over tissue perfusion, which was subsequently assessed sonographically and hence assumed a secondary role.

6 Low-dose Aspirin Co-treatment in Patients Undergoing IVF Treatment: Discussion

The 'aspirin group' comprised 46 couples and the 'placebo group' 51 couples. All couples invited for participation had consented to take part; but three couples who were randomized to the aspirin group later decided not to proceed with IVF treatment. There were no reported or observed side effects caused by the use of aspirin. For the extent of the study, there were no bleeding complications during oocyte collection procedures.

The strength of the study was its ability to establish cross-links between demographic, clinical, embryological, and ultrasonographic variables, Doppler indices and VEGF-VEGFR concentrations, to build a comprehensive model. All assessments were performed serially throughout the treatment for IVF on the same study cohort, allowing real time evaluation of different associations as they arose and interacted. A stepwise approach was employed for different clinical outcome variables in a logical time perspective from ET to positive pregnancy test, clinical pregnancy, and pregnancy beyond the first trimester. This was done as both a cross-sectional examination of the primary fresh IVF treatment cycles alone and a cumulative examination of the related FTER cycles.

The main weakness of the study was its small sample size, but wherever this has appeared to influence the level of significance of a trend, this has been specifically emphasized in the text. Because an extensive list of variables was analyzed in a relatively small study population, some of the associations and differences may be influenced by a random chance factor. However, the aim was, wherever possible, to interpret the results in an overall biologically rational model built with the support of other statistically significant findings.

The first part of the discussion follows, with comparison between the aspirin and placebo groups in terms of their demographic, clinical, and embryological variables and treatment outcome. The second part of the discussion covers the Doppler variables in terms of their association with the treatment outcome and the influence of aspirin on their performance. The third and final section of the

discussion is of VEGF and VEGFR interactions in the whole study population and separately in the aspirin and placebo groups.

6.1 Comparison of Aspirin and Placebo Groups

The demographic characteristics of the couples and the semen parameters of the male partners were similar in the aspirin and placebo groups. Furthermore, the two study groups received the same stimulation protocol (Day 21 vs Day 2), type of GnRH analogue, gonadotrophin and fertilization (conventional IVF or ICSI). The occurrence of ovarian cysts following down-regulation was similar in both groups *(Table 5.17-5.25CD).* The degree of technical difficulty and duration of the ET procedures were also similar in both groups. Hence, there was no confounding factor affecting the comparison of the two groups, that might be related to the ET procedure *(Table 5.33CD).* Therefore, with two study populations having a comparable background prognosis for assisted conception, any variation observed in the outcome of their treatment should be a function of that treatment. Consequently, the only variable with prognostic potential that differed between the two groups was the aspirin co-treatment.

No statistically significant differences in ovarian stimulation and *in vitro* fertilization variables were noted between the aspirin and placebo groups. Further, the number of embryos cryopreserved or transferred, and the anatomical grading of the best two embryos selected for transfer, did not differ. The percentage of couples having fresh ET, elective embryo cryopreservation due to the risk of OHSS, failed fertilization, and cycle cancellation before oocyte collection was also similar between the two groups *(Table 5.26-5.33CD).*

Therefore, in terms of clinical and embryological variables, no quantitative or qualitative improvement that can be attributable to the aspirin treatment was evident in the ovarian response to stimulation.

The ineffectiveness of the aspirin co-treatment was also witnessed in relation to endometrium and implantation. After fresh ET, the implantation and ongoing pregnancy rates were similar in the aspirin and placebo groups. Furthermore, no benefit of aspirin co-treatment was detected in minimizing biochemical

pregnancies or first trimester miscarriages. However, when the absolute values were studied beyond their statistical comparison, a different picture emerged. In the placebo group, the number of couples who achieved ongoing pregnancy was one third higher than that of the aspirin group (33% vs 24%). Although this difference was not statistically significant, a possible detrimental effect of aspirin on implantation cannot be ruled out (Table 5.6, *Table 5.26CD).* Because the ovarian response parameters were similar in both groups, even in absolute terms, it could be argued that if there is such a detrimental effect, it should be operating primarily on the endometrial aspect of the implantation process.

The same picture emerged when the cumulative outcome of the related FTER cycles were taken into consideration with the outcome of the primary fresh ET cycles (Table 5.7, *Table 5.26CD*). This cumulative outcome provides the opportunity for broader evaluation of the whole initial embryo cohort, beyond the outcome of the fresh cycle. Additionally, during the subsequent FTER cycles, no aspirin co-treatment was given to the women. Therefore, if any differences were noted in the outcome, they should have been due mainly to the presence of the aspirin effect on embryological factors or the absence of the aspirin effect on endometrial receptivity, assuming that the cryopreservation process does not eliminate the effect of aspirin on embryos. A limitation of this cumulative assessment is that it gives only a partial view, as not all the cryopreserved embryos created in the primary fresh IVF treatment study cycle were transferred by the time of this analysis.

The absence of any significant differences in this cumulative outcome assessment supported the initial observation from the primary fresh IVF treatment cycle, that there is no positive effect of aspirin on the embryo implantation potential or endometrial receptivity. However, absolute values of ongoing pregnancy still differed between the two groups in favour of the placebo group (35% vs 39%) but less strikingly than in the primary fresh IVF treatment cycle. This observation also provided some circumstantial evidence that once the effect of aspirin is removed from the endometrial development, as in FTER cycles, the presumed detrimental effect of aspirin on implantation becomes less

obvious, possibly suggesting the endometrium as the target organ more so than the ovary.

Contrary to the findings of the current study, Rubinstein et. al.[231], report that low dose aspirin co-treatment improves the utero-ovarian perfusion and that this is associated with a better response to ovarian stimulation in terms of number of mature follicles and number of oocytes collected, as well as better fertilization, implantation and pregnancy rates. These authors, like others in the literature, propose that a decrease in peripheral impedance in the uterine and ovarian vascular bed improves uterine receptivity and oocyte quality[115,244,229,525]. In the current study, no improvement in utero-ovarian perfusion at 150mg dosage was observed. This must be the critical factor affecting the outcome. In the absence of any perfusion effect, aspirin becomes ineffective in improving the probability of pregnancy and this is most likely a dose-dependent phenomenon. In their prospective, double-blind study, Rubinstein et. al.[231] use a similar protocol to the current study but with a lower-dose regimen of aspirin 100 mg/day.

Waldenstrom et. al.[249] report a modest increase in birth rate with aspirin compared with no treatment (27.2% vs 23.2%) giving an odds ratio of 1.2. Patients were randomly assigned to treatment with aspirin 75 mg daily from the day of ET until pregnancy test or no treatment. Hence, aspirin only affected the implantation process, but the confidence interval includes the unity.

Not all studies agree with Rubinstein et. al.[231] or with Waldenstrom et. al.[249]. In a prospective randomized study, with similar findings to the current study, Urman et. al.[240] report no differences in ovarian response but a lower clinical pregnancy rate in the aspirin group (39.6%) compared with the non-aspirin group (43.4%). This provides further support to the contention of the current study that implantation is hampered by aspirin and, although not exclusively, this is more significant than its probable influences over the ovaries. In a randomized study, Salman et. al.[243] report no difference between the aspirin and control groups in ovarian response parameters and pregnancy rates. Further, a prospective randomized, double-blind study reports no benefit from

80 mg aspirin supplementation per day in terms of ovarian and uterine blood flow or ovarian responsiveness in poor responders undergoing IVF treatment[250].

In their retrospective analysis, Tassa et. al.[242] also fail to demonstrate any improvement in ovarian response and pregnancy rates in women treated with low-dose aspirin (80 mg/day), and clinical pregnancy rates in the aspirin group are lower than in the placebo group (45% and 54%).

Other methods proven to increase tissue perfusion are also linked to better treatment outcome in IVF. Sher and Fisch[526] report that a NO donor (sildenafil) was effective in improving uterine artery blood flow and endometrial development with improved pregnancy chances. However similar interventions, if failing to improve tissue perfusion, were shown to be ineffective in improving the treatment outcome. In a population of women with a previous history of implantation failures and having treatment for IVF, Ohl et. al.[527] report that Nitro-glycerine (NTG) treatment on the day before ET is no more effective than placebo in improving the implantation or pregnancy rate and, as a NO donor, NTG treatment does not affect uterine Doppler values. In another study[528] women randomly received two sublingual spray emissions or placebo 3min before ET; NTG was used and no significant effect is reported on any parameter of the transfer procedure.

Therefore, the key point in response to such adjuvant treatments might be their ability to improve tissue perfusion. In populations where the intervention was not successful in improving tissue perfusion, the intervention was also ineffective and even maybe potentially detrimental to the ultimate treatment outcome. Why aspirin treatment may have such diverse effects on the outcome will be discussed in the following sections.

In the current study the platelet count and mean platelet volume, both before and after the ovarian stimulation, were similar in both groups *(Table 5.34-5.36CD)*. Therefore, it could be argued that the lack of observable aspirin effect in the ovarian and endometrial response parameters was because the aspirin dose used in the study was not optimized for its maximum anti-platelet effect.

However, the current study argued that it was not the antiplatelet effect of the aspirin that should be maximized but its vascular effect.

The benefit of aspirin is thought to derive from the inhibition of platelet Thromboxane-A_2 production[529]. Because Thromboxane-A_2 is a potent platelet aggregator and vasoconstrictor[530], the effect would be anti-thrombosis and this is thought to offset the thrombophilic tendency that leads to decreased tissue perfusion through platelet activation and arterial occlusion[531]. This antiplatelet effect of aspirin has found its successful clinical applications in cardiology, because the platelet-rich, intravascular thrombus is central to the pathogenesis of diminished coronary perfusion[532,533] and, to a certain extent, antiplatelet effect also explains the success of aspirin treatment in recurrent miscarriage due to antiphospholipid antibody syndrome. In the latter condition, an exaggerated haemostatic response leading to thrombosis of the uteroplacental vasculature has been suggested as the underlying pathology along with the direct trophoblastic dysfunction. In such women, pregnancy outcome appears to improve with aspirin alone[196]. An even greater improvement is reported with the concomitant use of heparin[196,197]. Conversely, if there is no thrombophilic tendency, aspirin is not useful in the prevention of recurrent early pregnancy losses[534].

However, an autoimmune mediated hyperthrombotic state did not find much scientific evidence in the pathogenesis of decreased fertility. Despite a higher prevalence of antiphospholipid antibodies, the IVF population did not appear to be affected negatively with these antibodies contrasting to recurrent miscarriages[218]. On the contrary, diminished uterine perfusion has been a widely accepted pathology of subfertility, indicating that what needs to be corrected is not an intravascular, but rather a vascular pathology[252].

Aspirin also inhibits cyclo-oxygenase enzymes in the vascular endothelium, which is the source of prostacyclin, a potent inhibitor of platelet aggregation and a vasodilator[535] with the effect then being thrombogenic. Nevertheless, the selective inhibition of platelets sparing the functional capacity of endothelium is possible (refer to section 1.2.2). This can alter the prostacyclin: thromboxane

ratio favourably and result in increased tissue perfusion. As the effect occurs through lowering of the vascular tone, it is independent from the state of platelet activation. It is this vascular effect of aspirin that should be prioritized in subfertility patients.

At higher doses aspirin can achieve more complete and rapid platelet inhibition, and at lower doses can spare more endothelial cyclooxygenase activity. In this context, using higher doses of aspirin maximizes the anti-platelet effect, and this is only beneficial if the tissue perfusion is primarily hampered by intravascular micro-thrombus formation. On the contrary, where tissue perfusion is compromised by high vascular tone, higher doses of aspirin are detrimental and lower doses should be used to achieve vasodilatation. Aiming for a higher PgI_2:TxA_2 ratio with low-dose aspirin is a sub-optimal compromise in recurrent miscarriage patients, as it does not improve tissue perfusion or pregnancy outcome as much as high doses can achieve[162,536]. Aiming for a higher anti-platelet effect in IVF patients may be equally detrimental as it will lower the PgI_2:TxA_2 ratio and subsequent tissue perfusion.

Further to its vascular effect, aspirin may have far reaching effects on implantation. Human endometrium secretes a variety of prostaglandins, including the stable metabolites of prostacyclin and thromboxane A_2: 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2[236,537]. An unfavourably altered prostacyclin:thromboxane ratio and prostaglandin production may increase the probability of expulsion of the blastocyst and hamper the preparation of the endometrium for implantation[234,235,236]. Embryo-endometrial communication and decidualisation has been linked to prostaglandin stimulated inflammatory process and the release of interleukins[232,233]. Aspirin inhibition of local prostaglandins may impede this process and lower the implantation rate.

High doses of salicylates are usually inhibitory to kinases. Targets include pro-inflammatory enzymes, cytokines, chemokines, and cell adhesion molecules. COX-2 over-expressing cells produce prostaglandins and proangiogenic factors, and stimulate both endothelial migration and tube formation. This effect is inhibited by aspirin[156]. Hence, while tissue perfusion was improved via

existing vasculature either at lower doses through vascular effect or at higher doses through anti-platelet effect, depending on the underlying pathology, in a dose-dependent manner aspirin can actually interfere with the new vessel formation, which is far more fundamental to folliculogenesis, endometrial development and implantation.

In the current study, the mean platelet volume after ovarian stimulation was insignificantly higher in the non-pregnant women than the pregnant women who all received aspirin co-treatment. This may signify a lower probability of pregnancy in women whose biological response to aspirin was higher, in terms of anti-platelet effect. This provided further circumstantial evidence that aspirin co-treatment might be detrimental to the IVF outcome at the dose used in this study.

It can be concluded that the optimal dose influencing vascular haemostatic regulation depends on the balance between the anti-platelet and vasodilatory effects of aspirin, and dose selection should be based on the reasons for impaired perfusion.

In summary, no beneficial effect of aspirin at the current dose regimen was found in the current study in the outcome of fresh IVF treatment. Despite no differences in the ovarian response to stimulation and subsequent embryo implantation and ongoing pregnancy rates, a legitimate argument has emerged that aspirin might act detrimentally on the endometrium during implantation, because the dosage used in the current study was probably high enough to compromise the optimum prostacyclin:thromboxane ratio required for better tissue perfusion. The absence of any significant improvement in the utero-ovarian tissue perfusion in the aspirin group also supports this contention.

Implantation is a complex, closely regulated, highly selective but poorly understood process. Its vital role in the survival of species demands this meticulous complexity to select the fittest and to ensure the sound foundations of the best environment for the selected embryo during the forthcoming most demanding nine months. Therefore, it is not surprising to encounter

unprecedented and even conflicting results of any intervention that is not precise enough to pinpoint specific steps of this interlinked process without disturbing the others. Certainly aspirin is far from being a specific modulator with an ever increasing list of actions, and interactions.

6.2 Doppler Assessment of Utero-ovarian Perfusion

In an unselected subgroup of women undergoing IVF treatment and taking part in the 'aspirin study', uterine artery blood flow, sub-endometrial blood flow, peri-follicular vascularity and vascular impedance were assessed. Multi-point assessment in reference to time and site allowed the development of a physiological framework to understand the harmonious efforts of the ovary and uterus during assisted conception. The study was powered to evaluate the relation between uterine artery impedance values and ongoing pregnancy rates. Therefore, some of the observed trends of association fell short of statistical significance and should be interpreted cautiously, in view of the relatively small sample size for sub-endometrial and follicular perfusion.

There were no differences in demographic characteristics between pregnant and non-pregnant women in terms of age, parity, number of previous fertility treatments, BMI and basal FSH values (Table 5.8-5.10). This allowed like-to-like comparison of the clinical and sonographic responses to IVF treatment.

The first assessment of uterine artery and sub-endometrial spiral artery blood flow was carried out after pituitary down-regulation, which removed the effect of follicular and endometrial angiogenesis and represented the baseline state of pelvic perfusion. Scans on the last day of stimulation were then able to reflect the true response potential of the individual under ovarian stimulation.

A single operator performed the ultrasound scans in order to eliminate inter-observer variances. Serial assessment of the peri-follicular blood flow was achieved with hard copies of the scan pictures. This was not possible for the measurement of the sub-endometrial spiral artery impedances. However, vessels with the maximum Colour Doppler intensity were chosen for the representative evaluation. The calibre and anatomic localization of the uterine

arteries, with respect to sonographically detectable landmarks, allowed the longitudinal assessment of the uterine artery impedance values. As no differences were found in mean Doppler indices of the right and left ascending uterine artery, further analysis was carried out with their mean values *(Table 5.39, 5.40CD).* Because a strong correlation was found between pulsatility index (PI) and other Doppler indices (RI, S/D), PI was used for the comparative analysis with the advantage of reflecting blood flow impedance accurately even in the absence of end-diastolic blood flow.

A strong correlation was detected between the uterine, sub-endometrial and follicular blood flow *(Table 5.41-5.44CD).* However, the predictive values differed in determining the treatment outcome and discriminating the good prognostic group. Although no difference was found between the pregnant and non-pregnant groups in the uterine artery impedance during the baseline scan, in the pregnant group, the difference gradually widened and reached statistical significance by the day of the last ultrasound scan (Table 5.11). Therefore, it was not only the static cross-sectional observation but also the dynamic trend of these parameters that presented a prognostic value.

As the uterine artery impedance values were prognostically linked to the treatment outcome, a cut-off point was selected to facilitate its clinical use in the selection of good and poor prognostic groups. Because the data proposed a high negative predictive value, the cut-off point was selected to discriminate the poorest prognostic group. A cut-off point of 3 for uterine artery PI indicated a negligible chance of achieving pregnancy (Table 5.17).

This finding was in agreement with other trials reporting similar cut-off points of uterine artery PI values with high negative predictive power[115,226,256,260]. However, the literature is contradictory as others report no significant differences in uterine artery PI values between conception and non-conception cycles[278,280,283,290,321]. Differences in stimulation protocols, study groups, timing of Doppler investigations and study designs, either cross-sectional or longitudinal, appear to affect the outcome and could be considered as the possible explanation for these conflicting results.

Perifollicular vascular impedance was found to be lower in the pregnant group on the last day of stimulation (Table 5.12). Although this trend did not reach statistical significance in two-tailed hypothesis testing, a cut-off point of 1.08 for PI values was shown to discriminate the women with good and poor prognosis (Table 5.19). The discriminative performance of the test reached statistical significance at one-tailed hypothesis testing, employed because the published data had already established the direction of the association between follicular perfusion and the treatment outcome[319,322,324].

Perifollicular vascularity, defined as the percentage of follicular circumference that showed contact vascularity with visible flow, was semi-quantified using a subjective scale[321,322]. No correlation was detected between the degree of perifollicular vascularity and the vascular impedance expressed as PI. There were no differences in the peri-follicular vascularity values between pregnant and non-pregnant women (Table 5.12).

These results were contradictory to the findings of Bhal et. al. and Coulam et. al., and may be explained by the differences in patient population, the effect of different stimulation protocols, and differences in the design of the current study[321,322]. In the current study, the perfusion of a single follicle having the most extensive vascularity on power Doppler mapping was evaluated, whereas in the previously mentioned studies, multiple follicles are assessed. Furthermore, in the current study, the majority of cases remained in the low vascularity range (<25% of contact vascularity). This was not surprising, because all of the Doppler assessments were performed prior to the luteogenesis of hCG administration. The physiology of limited vascularization during follicular development, as opposed to intense neovascularization of the corpus luteum, might hinder the detection of significant differences with prognostic importance. Therefore, the timing of the assessment in reference to hCG administration, might explain the lack of association between the degree of vascularity and the treatment outcome.

Nevertheless, the current study findings suggested that it was the vascular impedance rather than the degree of vascularity that predicted the treatment outcome, and this allowed the hypothesis that the amount of blood flow within a given artery determined the tissue perfusion better than the contact surface of the vessel to the tissue of concern. Furthermore, as vascularity depicted by the semi-quantitative assessment of the power Doppler mapping cannot differentiate arterial and venous supply from each other, it makes physiologic sense to assume that quantitative Doppler evaluation of the arterial flow correlates better with the perfusion related outcomes. The quantitative assessment was based on PI values, which took into consideration the diastolic as well as systolic compartment of the flow in an angle independent equation, since it has been shown that peak systolic velocity of peri-follicular blood flow is not prognostically significant[323].

When the data were evaluated to identify the clinical characteristics associated with high peri-follicular vascular impedance, these cycles were distinguished by their lower oestrogen concentrations and lower number of mature follicles, oocytes, and 2-PN embryos (Table 5.23). These findings agreed with the study of Battaglia et. al.[362] and provided evidence of an association between poor follicular perfusion, poor ovarian response and poor clinical outcome.

The pathophysiology of this clinical association can be explained from a genetic perspective. Huey et. al. report that PI is significantly and negatively correlated with follicular fluid O_2 concentration and the same correlation is reported between RI and the fertilization rate of oocytes[342]. Van Blerkom et. al.[107] relate the developmental potential of oocytes to the dissolved oxygen content of follicular fluid. The mechanism is explained by chromosome mis-segregation in hypoxic follicular milieu.

Vascular impedance of the sub-endometrial spiral arteries showed a progressive change towards lower PI values during ovarian stimulation and this drop in resistance was more evident in the pregnant than non-pregnant women. However, this trend fell short of statistical significance (Table 5.13).

Yuval et. al. report similar findings, where Doppler indices of endometrial blood flow show a non-significant trend towards lower impedance values in the pregnant group[274]. Zaidi et. al.[293] report no significant difference between pregnant and non-pregnant groups with regard to sub-endometrial peak systolic blood flow velocity or sub-endometrial PI, except for the absence of flow predicting poor probability of pregnancy. Likewise, endometrial vascularization with power Doppler assessment is reported to have no prognostic value in an ICSI programme[304]. However, Schild et. al.[282] who used three-dimensional power Doppler sonography and Yang et. al.[291] who evaluated intra-endometrial power Doppler area, report significantly higher pregnancy rates in women with better endometrial perfusion. Battaglia et. al.[229] also report that PI values on the day of oocyte collection are significantly lower in patients who become pregnant.

Differences in the published literature can be attributed to relatively small-sized study groups, which failed to demonstrate an existent relationship between (sub)endometrial blood flow and endometrial receptivity. In the current study, the high coefficient of variance leading to extensive overlap between the two outcome groups supported this power problem. Despite the physiological role of local tissue perfusion in the functional capacity of organ systems, variability between the published studies indicates a current inability to identify the right method of assessment of low velocity blood flow in the endometrium. Three-dimensional power Doppler ultrasonography may provide more accurate means of evaluating such vascularization.

Neither endometrial thickness nor its echo-pattern had prognostic value in the prediction of treatment outcome. Although endometrial thickness progressively increased during ovarian stimulation this was comparable in both the pregnant and non-pregnant group. This growth was also found to be independent of the level of uterine and sub-endometrial vascular impedance and serum oestradiol levels. Similarly, regardless of their treatment outcome, all women presented with a multi-layered triple-line endometrium (Table 5.15, 5.16).

Related data from the literature are conflicting. While an association between endometrial thickness and E_2 levels is reported[428,538] other studies dispute such correlation[388,389,404]. Differences can be explained on the basis of timing of assessment during the ovulation induction[397].

It is possible that supraphysiological concentrations of oestradiol achieved in fresh IVF treatments were well above the required stimulation levels for endometrial growth, so that differences above this level make no change in the growth pattern of endometrium. In such hormonal milieu for anatomic growth, it may be the paracrine control that determines the functional differentiation and receptivity.

The published literature is also inconclusive with regard to the value of endometrial thickness and echo-pattern. While the current study on endometrial echo-pattern agreed with some[256,397,398,403,420,421]; others report significant association between endometrial echo pattern and the outcome of IVF treatment[257,382,383,392,393,396,390,404,405,418,539].

Similarly, the prognostic value of endometrial thickness in terms of pregnancy rates is not universally accepted. Whilst a lack of association is reported by some[143,256,257,283,395,396,397,398,402,403,408,420,421,540,541], others report a significant association between endometrial thickness and treatment outcome[382,388,390,393,423,542]. To the contrary, Weissman et. al. report that implantation and pregnancy rates are significantly reduced in patients with endometrial thickness of >14 mm on the day of hCG administration[543].

Because of the differences in stimulation protocols, and timing and methods of sonographic assessment of the endometrium, a definitive conclusion is not possible but, as the endometrial thickness and echo-pattern reflects the supraphysiological hormonal milieu of ovarian stimulation, threshold stimulation is likely to be achieved even with a poorly receptive endometrium in terms of anatomical growth. Nevertheless, the available data suggest that at present the sonographic and Doppler assessment of endometrium in terms of functional capacity and fertility potential is not optimized for routine clinical use.

The current study found no correlation between the vascular impedance values of the uterine arteries and corresponding oestradiol, FSH and BMI values (Table 5.14). This finding confirmed previously published data[425]. Although both PI and oestradiol values showed progressive and opposite directional changes during ovarian stimulation, their lack of correlation proposed a non-hormonal modulation of the uterine perfusion. Although basal FSH and BMI values had a wide range of 30.30 IU/l and 19 kg/m^2 respectively, their mid-central distribution was narrow with an inter-quarter range of 3.4 and 5 respectively. Therefore, as both basal FSH and BMI values were predominantly within the normal range, it was unlikely that they could have explained the overall variation of the vascular impedance values, which had a wider distribution on either side of the chosen cut-off point.

In summary, the current study provided evidence for the association between utero-ovarian perfusion and reproductive outcome following IVF treatment. In women with PI values >3, the probability of achieving pregnancy was negligible when predicted by the last day uterine artery Doppler measurement. Likewise, in women with perifollicular PI values >1, the probability of achieving pregnancy was insignificant. Therefore, if Doppler assessment reveals impedance values above these cut-off points, the created embryos could be cryopreserved for subsequent FTER cycles. However, this approach is unlikely to overcome intrinsic embryo related problems. Furthermore, it may actually reduce the probability of pregnancy due to detrimental effects of cryopreservation. Measures to decrease uterine and ovarian vascular impedance reflected by lower PI values might enhance pregnancy rates by improving embryo quality and uterine receptivity for implantation. As vascular indices were not associated with higher E_2 levels, non-hormonal methods such as omega-3 fatty acid supplementation, NO donors, or future angiogenetic agents with regional/topical application could provide alternatives.

6.2.1 Comparison of Doppler Indices of the Aspirin and Placebo Groups

At a daily dose of 150mg, aspirin did not improve uterine artery, sub-endometrial, and peri-follicular blood flow, or the perifollicular vascularity and endometrial thickness. This observation was valid after both pituitary down-

regulation when hormonal effect over vascular tonus was minimal, and during ovarian stimulation with increasing oestradiol concentrations *(Table 5.46CD).*

This lack of effectiveness over tissue perfusion was most likely to be a dose dependent phenomenon secondary to the loss of selective inhibition of platelet cyclooxygenase, while sparing the functional capacity of endothelium, thereby altering the prostacyclin:thromboxane ratio unfavourably and resulting in loss of beneficial effect on vascular tonicity and tissue perfusion.

With repeated administration, acetylation of cyclooxygenase by oral aspirin is dose-dependent, cumulative and selective. However, biochemical selectivity is at best relative, not absolute, and partial inhibition of prostacyclin formation seems to be an unavoidable consequence of inhibition of platelet cyclo-oxygenase by aspirin. With single daily administration, the aspirin dose for near-complete suppression of serum thromboxane formation with best differential sparing of prostacyclin, has been titrated downward to approximately 0.5-2 mg/kg[157]. It appears that a mean >40 mg/day of aspirin is necessary for plateau inhibition of platelet aggregation, although suppression of prostacyclin will inevitably be more pronounced. Fitzgerald et. al.[159] report that depression of Thromboxane-metabolite attained statistical significance at doses of aspirin ≥80 mg/day, with the fall in PGI-metabolite excretion attaining significance at doses of aspirin >160 mg/d. Therefore, at 150mg/day the current study may iatrogenically have diminished the theoretical benefit aspired to by low dose aspirin in tissue perfusion.

6.3 VEGF-VEGFR Concentrations

Pre-treatment serum samples were studied for VEGF and VEGFR levels in 80 women during natural cycles prior to the IVF treatment. On the day of oocyte collection 59 post-treatment serum and 55 follicular fluid samples were also available. Thirty-six pre-treatment serum samples, 29 post-treatment serum samples and 27 follicular fluid samples were from the aspirin group (Table 5.24).

There was a positive correlation between pre-treatment and post-treatment serum VEGF levels and this was also valid for serum VEGFR levels. VEGF exhibited a negative correlation with its receptor both before and after the treatment. Contrary to this, follicular fluid VEGF correlated positively with its receptor. No correlation was found between serum and follicular fluid VEGF levels *(Table 5.54CD)*. In terms of angiogenetic response, positive correlation between pre- and post-treatment serum samples reflected the importance of baseline characteristics that determined the boundaries of personal response to ovarian stimulation. In other words, women who presented with a favourable angiogenetic steady state were able to provide even more favourable angiogenetic support to folliculogenesis and endometrial development during IVF treatment. Women with a less favourable angiogenetic background were limited in their angiogenetic response by their disadvantageous starting point.

The negative correlation between VEGF and VEGFR levels in serum suggested a dynamic equilibrium between these two systems, probably working in opposite directions. In support of this contention is the report of He et. al.[365] that alternative splicing of the VEGFR-1 mRNA produces soluble VEGFR-1 which significantly contributes to the regulation of VEGF activity. Rising sVEGFR-1 production is reported to be detrimental to follicular development due to diminished bio-active VEGF supply[544].

However, the direction of association between VEGF and VEGFR levels was the same in follicular fluid, possibly indicating that different mechanisms determined the levels of these two factors in systemic circulation and follicular fluid. Supporting this contention, the current study found no link between serum and follicular fluid concentrations.

The following is a set of proposals for biologically plausible VEGF-VEGFR interactions on the premise that hypoxemia enhances VEGF expression in maturing follicles and in the endometrium[360,545]; while VEGFR diminish bio-active VEGF supply[544].

VEGF response can be described as 'U-shaped'. High levels could indicate a state of optimum stimulus for angiogenesis resulting in better tissue perfusion and consequently higher oxygen tensions, but at the same time higher levels could also indicate a physiological response to a hypoxic state. Therefore, an isolated high VEGF measurement could have been associated with either hypoxia or normoxia. Lower VEGF levels could indicate a state of normal tissue oxygen tension or, to the contrary, the absence of angiogenetic response to a hypoxic stimulus. Therefore, an isolated low VEGF concentration could indicate either good tissue perfusion or inability of tissue to generate its angiogenetic stimulus to hypoxia.

To demonstrate this dichotomy, some reports suggest that VEGF is associated with high vascularization and oxygenation, resulting in oocytes with superior pregnancy potential[107,348,356,357,358], while others demonstrate that an elevated level of VEGF implies follicular hypoxia[359,360], thus considered a marker of diminished pregnancy potential[349].

Concomitant measurement of VEGF and VEGFR levels may be helpful to differentiate both states. If both VEGF and VEGFR levels are low, the absence of stimulation could be argued as both hypoxia and VEGF itself positively regulate VEGFR expression, which in turn controls VEGF-driven angiogenesis[546]. The absence of stimulation could be due to the presence of an optimum physiological state and in this situation outcome of the physiological state should be desirable, such as in higher ovarian response or successful implantation. Alternatively, the absence of stimulation could be due to the inability of the tissue to respond to an unfavourable situation and the outcome would be undesirable, such as in poor ovarian response or implantation failure. If VEGF and VEGFR levels are both high then the presence of a stimulated state and a counter regulatory response of VEGFR to prevent over stimulation by increasing VEGF levels could be argued. It is already discussed in the literature that the soluble form of VEGFR, which is free in circulation or follicular fluid, acts as a deterrent factor against VEGF activity by blocking it to stimulate the membrane bond physiologically active receptors. If VEGF levels are high and VEGFR levels are low this logically indicates a state of maximum

stimulation which, at the time of sampling, has not reached the favourable steady state. Alternatively if VEGF levels are low and VEGFR levels are high the possible conclusion is over-correction of angiogenetic stimulation or indeed a state of inappropriate suppression that may eventually lead to hypoxia.

The current study investigated the physiological role of VEGF and VEGFR by associating their concentrations to different treatment outcomes. The first outcome measure was the ET.

Pre- and post-treatment serum VEGF and VEGFR concentrations were statistically similar in women who had ET and those who did not. Follicular fluid VEGF concentrations were also similar in these two groups but follicular fluid VEGFR concentrations were significantly lower in women with no-ET. In the current study the most common reason for no-ET was the imminent risk of OHSS and so the lower follicular fluid VEGFR concentration was likely to reflect a high responsive state in ovarian stimulation *(Table 5.47-5.52CD).*

Unlike the follicular fluid measurements, the absence of statistically significant differences in serum samples indicated the importance of obtaining samples from the target environment as, despite having persistently low VEGF and VEGFR levels in all serum samples from the no-ET group when compared with the ET group, the differences became insignificant once they were diluted in the systemic circulation. Although there was no such dilution effect on follicular fluid VEGF concentrations, the lower values in the no-ET group did not reach statistical significance. This may have been due to small sample size limiting the differences from attaining significance beyond a chance factor.

Because on this occasion, the outcome of ovarian stimulation was favourable, lower levels of VEGF and VEGFR could not have been due to under-stimulation but rather indicated a state of favourable oxygenation not requiring VEGF stimulation and subsequent compensatory VEGFR elevation. In this setting, higher VEGF and VEGFR levels probably indicated poor oxygenation.

To verify the association between high ovarian response potential and low follicular fluid VEGFR levels, the no-ET group was analysed. Follicular fluid VEGFR concentrations were particularly lower in women with elective no-ET due to the imminent risk of OHSS, when compared with the ET group. Once again, no significant differences were found in pre- and post-treatment serum VEGF and VEGFR levels in all outcome groups: ET, no-ET due to elective cryopreservation of all embryos, failed fertilization or cancellation before oocyte collection. Follicular fluid VEGF levels were also similar in these groups *(Table 5.47-5.52CD)*.

The second set of outcome measures compared was clinical pregnancy, no-pregnancy and no-ET. Follicular fluid VEGFR concentrations were significantly lower in the no-ET group, as already observed in the previous analysis. However, when compared with the non-pregnant group, insignificantly lower follicular fluid VEGFR concentrations were evident in the pregnant group and the same was noted for ongoing pregnancy. Follicular fluid VEGF levels were also lower in the no-ET and the pregnant group but at a statistically insignificant level. No differences were detected between the pregnancy, no-pregnancy and no-ET groups in terms of pre- and post-treatment serum VEGF and VEGFR concentrations *(Table 5.47-5.52CD)*. Therefore, the argument detailed in the ET analysis also applied in this analysis, where insignificantly low follicular fluid VEGF and significantly low VEGFR concentrations in the good prognostic group, indicated a state of good tissue perfusion and oxygenation rather than inability to provide a physiological stimulation against hypoxia.

Failure of low follicular fluid VEGF levels to reach significance was due either to the inability of the good perfusion group (pregnancy) to lower their VEGF levels or, more possibly, the inability of the poor perfusion group (no pregnancy) to increase their VEGF levels against hypoxia, so that in either case the difference was not large enough to attain significance. Alternatively, in the poor perfusion group, significantly high follicular fluid VEGFR concentrations arguably sequestrated the already high levels of VEGF initially stimulated by hypoxia, and subsequently made lesser amounts of free VEGF molecules available for ELISA test detection; thereby, the difference failed to reach significance at the

time of sampling. In this scenario, follicular fluid VEGFR concentrations were inappropriately but significantly high for the level of hypoxia leading to an unfavourable outcome.

The data were further evaluated to investigate whether any of the VEGF-VEGFR variables could differentiate ongoing pregnancy from miscarriage or biochemical pregnancy, following the fresh IVF treatment cycle. No differences were found between these groups in terms of pre- and post-treatment serum VEGF and VEGFR levels or their follicular fluid concentrations. Although lower follicular fluid VEGFR levels indicated a state of higher ovarian response and a trend of better probability of pregnancy, it appeared that its prognostic role regarding the outcome of implantation (chemical pregnancy, miscarriage, or ongoing pregnancy) was not as significant *(Table 5.51CD).*

No significant correlation was found between the number of implanted embryos following fresh treatment and any of the serum or follicular fluid VEGF-VEGFR levels. To provide a wider assessment of the whole embryo cohort created during the fresh treatment, the cumulative implantation figures including both the fresh and related FTER cycle were evaluated. In this assessment, the number of embryos implanted correlated negatively with follicular fluid VEGF levels at a significant level *(Table 5.53-5.56CD).*

To clarify whether follicular fluid VEGF levels differ on a continuous scale with the increasing number of implantations, a comparison was made of concentrations between the no-implantation, singleton implantation, and twin implantation groups. Follicular fluid VEGF concentrations showed a noticeable variance only between the no-implantation group and the rest of the cohort, indicating that higher levels are associated with poor outcome but levels do not progressively drop with increasing numbers of implantations.

Neulen et. al.[366] define an index of biological activity of VEGF by VEGF:sVEGFR-1 ratio and show an increasing availability of VEGF with higher ovarian response to gonadotrophin therapy. However, in the current study, the VEGF:VEGFR ratio, level of change in VEGF concentrations during IVF

treatment (pre-treatment minus post-treatment) and the categorical expression of this change (decreased or increased) showed no difference in the outcome variables *(Table 5.57, 5.60CD).*

6.3.1 Factors Linked to VEGF-VEGFR Levels

After realizing the positive association between low follicular fluid VEGF and VEGFR levels and the desirable treatment outcome in terms of ovarian response, and possibly implantation and pregnancy, in order to propose a model by which VEGF-VEGFR may affect the treatment outcome, the demographic, clinical and embryological variables persistently linked to the lower or higher VEGF-VEGFR levels were investigated. In this assessment pre-treatment serum concentrations reflected the baseline characteristics of the women and post-treatment serum concentrations reflected their response to ovarian stimulation built on the pre-treatment background of steady state values *(Table 5.61-5.70CD).*

Pre- and post-treatment serum VEGF and VEGFR levels did not show a common theme where the associations with demographic variables revealed a good or poor ovarian reserve. However, higher follicular fluid VEGF and VEGFR levels indicated a lower ovarian reserve, as they positively correlated with female age; follicular fluid VEGF also correlated positively with female weight and BMI. Therefore, any future studies or clinical applications of follicular fluid VEGF should use a BMI-correction in comparative analysis *(Table 5.61CD).* No significant association was noted between VEGF-VEGFR concentrations in serum and follicular fluid, or menstrual cycle regularity, female smoking, alcohol consumption, and type and aetiology of the subfertility.

It was not possible to make inferences for the pre- and post-treatment serum VEGF concentrations in terms of ovarian response because the level of associations either did not reach significance or did not reveal a biologically plausible pattern *(Table 5.69CD).* However, it was of note that post-treatment serum VEGF concentrations still showed similar associations as revealed by pre-treatment levels, once again reflecting the dominant and persistent nature of the baseline predilections throughout the ovarian stimulation. Similarly pre-

and post-treatment serum VEGFR did not correlate with any of the clinical and embryological variables tested at a significant level but the direction of the associations appeared to be the opposite of that of the corresponding VEGF levels. These also provided evidence of the initial contention that VEGF-VEGFR systems possessed opposing and probably balancing effects.

High follicular fluid VEGF and VEGFR concentrations once again portrayed a picture of poor ovarian reserve characterized by the need for more intense ovarian stimulation and subsequently poor response to this stimulation. They not only correlated positively with female age, but also with the initial gonadotrophin dose, duration of ovarian stimulation, total gonadotrophin dose required for ovarian stimulation, and negatively with the oestradiol level before hCG administration, the number of follicles measuring 10-15mm and 16-17mm, number of follicles aspirated, number of oocytes collected and used for fertilization, number of 1-PN, 2-PN and 3-PN embryos created, and number of embryos cryopreserved. There was a tendency of an association between high follicular fluid VEGFR concentrations and high FSH levels (p: 0.09).

It was of major interest that no correlation was detected with embryo quality. Previous studies by Van Blerkom et. al.[107,357] report a link between follicular fluid VEGF levels and subsequent embryo quality, based on the argument that hypoxic follicular environment leads to chromosomal abnormalities of the dividing oocytes and consequently poor fertilization and embryo growth.

In the current study, there had been a consistent and significant association of high follicular fluid concentrations of VEGF and VEGFR and poor ovarian reserve, poor ovarian response to stimulation and poor treatment outcome, that in turn were associated with older female age and higher BMI.

6.3.2 Associations between VEGF- VEGFR Levels and Doppler Indices

The association between VEGF and VEGFR concentrations and pelvic perfusion assessed by Doppler ultrasonography was investigated (Table 5.31). In the whole study population, a tendency was noted to an association between poor uterine artery and sub-endometrial perfusion during IVF treatment (high PI

values ± low drop in PI values during treatment) and higher pre- and post-treatment serum VEGF, lower pre- and post-treatment serum VEGFR level, and higher follicular fluid VEGF and VEGFR levels. These observations provided further evidence that high VEGF levels are the hypoxic response of the system, as they are likely to be associated with poor perfusion. This hypoxic response in women with poor uterine perfusion was a personal prognostic characteristic evident at the baseline investigation but this also persisted throughout the treatment for IVF and was consequently observed at the end of the ovarian stimulation, both systemically in the serum and locally in the follicular fluid. Serum VEGFR levels also responded to this hypoxic state and were maintained at a lower concentration to maximize the availability of the free VEGF form to bind the membrane receptors. However, in follicular fluid higher VEGFR levels were found as a reflection of poor uterine-endometrial perfusion indicating that either there was an effort to control biological activity of the rising VEGF levels during hypoxia or inappropriately high follicular fluid VEGFR levels were actually preventing the available VEGF from improving the tissue perfusion at the time of sampling and leading to hypoxia.

As already documented, good uterine perfusion detected by Doppler analysis was associated with better sub-endometrial and follicular perfusion and better ovarian response to stimulation and higher pregnancy rates (Table 5.17, 5.21, *Table 5.41-5.44CD).* It was also shown that good uterine and sub-endometrial perfusion was associated with low follicular fluid VEGF and VEGFR, which were also associated with good ovarian reserve, better ovarian response to stimulation and possibly better implantation and pregnancy rates.

The initial model of the current study stated that low VEGF and VEGFR levels have indicated optimum oxygenation and desirable outcome, and low VEGFR levels in the presence of high VEGF levels have suggested a hypoxic response. The observations of the current study fitted this simple model.

Lee et. al.[345] show that follicular fluid VEGF concentrations are more than 10-fold greater than serum concentrations at the time of oocyte collection. Manau

et. al.[347] also detect significantly higher follicular fluid concentrations of VEGF than the corresponding circulating concentrations.

A positive correlation is reported between blood VEGF levels and the number of follicles detected by vaginal sonography prior to oocyte collection[352,353,354]. In contrast, Manau et. al.[347] observe no relationship between circulating concentrations of VEGF and parameters of ovarian response to gonadotrophin stimulation on the day of oocyte aspiration.

Although some reports suggest that high follicular fluid VEGF levels are associated with high follicular vascularization and oxygenation, resulting in oocytes with superior pregnancy potential[107,348,356,357], other studies show that an elevated level of follicular fluid VEGF implies follicular hypoxia[345,347,359,360]. Thus, the increased follicular fluid VEGF concentration is considered a marker of diminished pregnancy potential[349,361] and correlates negatively with embryo quality in IVF patients[547]. Likewise, Battaglia et. al.[362] report that poor responders experience over-production of follicular fluid VEGF consistent with ovarian ageing and decreased ovarian reserve. Quintana et. al.[363] observe that follicular fluid VEGF concentration is elevated in patients with decreased ovarian response to controlled ovarian hyperstimulation. Tokuyama et. al.[548] report that regardless of the stimulation protocol, women with the highest number of oocytes collected have the lowest VEGF concentrations in follicular fluid.

Barroso et. al.[547] describe no significant association between blood flow indices and follicular levels of VEGF, unlike Van Blerkom et. al.[107,357] who show higher VEGF levels in follicles with higher dissolved oxygen contents and with Doppler findings indicative of increased blood flow.

All of these findings give credence to our contention that in the absence of simultaneous VEGF-VEGFR measurements, isolated assessment of VEGF levels could lead to conflicting results and conclusions. The observations and interpretation of VEGF levels in the current study, extended by the combine VEGFR interactions could provide a physiologically plausible model.

6.3.3 Aspirin and VEGF-VEGFR Levels

In the following section the aspirin-VEGF-VEGFR interaction will be discussed (Table 5.25). Differences in pre- and post-treatment serum and follicular fluid levels of VEGF and VEGFR were not statistically significant. However, a biologically plausible model may be inferred by the tendencies observed in the absolute values of VEGF-VEGFR levels.

Serum and follicular fluid VEGF levels were persistently lower in the aspirin group when compared with the placebo group both before and after IVF treatment. Serum VEGFR levels, although higher in the aspirin group prior to treatment, was level with that of the placebo group in the post-treatment samples. In the aspirin group, much lower follicular fluid VEGFR levels were found than in the placebo group.

Therefore, with the limitation of a significant chance factor in mind, such a distribution of differences in aspirin and placebo groups might have indicated a subtle tendency towards better perfusion in the aspirin group. Doppler assessment differences between the aspirin and placebo groups were mostly in favour of the aspirin group with lower vascular resistance, but once again were not statistically significant. Hence, these two separate assessments (biochemical and physical) of the same condition (tissue perfusion) agreed in broad terms but were not strong enough to reach a level of statistical significance beyond the pre-set level of an acceptable chance factor.

In this context, it is of interest to refer to the observations of Tsuji et. al.[156] which provide an alternative scenario; where aspirin can slow down the endothelial migration and tube formation stages of neovascularization via inhibition of COX-2 and subsequent proangiogenic factor production. Hence, lower concentrations of VEGF both in follicular fluid and in peripheral circulation could be the direct result of aspirin. VEGF stimulates nitric oxide release from endothelial cells[331] and induces the release of prostacyclin by activating cytosolic phospholipase A_2, causing the release of arachidonic acid[332]. Both of these actions would be expected to promote vasodilatation. Aspirin can irreversibly block this process.

6.3.4 Prognostic Significance of Doppler Variables of Utero-ovarian Blood Flow and VEGF and VEGFR in Serum and Follicular Fluid in Prediction of Pregnancy

On an extensive list of demographic, clinical, embryological, Doppler, ultrasonographic and VEGF-VEGFR variables, logistic regression analysis highlighted the uterine artery Doppler indices in the late follicular phase of ovarian stimulation and post-treatment serum VEGFR levels as the only independent prognosticators. This finding indicated the fundamental role of tissue perfusion whether expressed in physical terms by Doppler assessment or in biochemical terms by VEGF-VEGFR levels in the fertility potential of the women, leaving the demographical and clinical variables as surrogate markers. Furthermore, it was of note that both qualitative and quantitative embryological variables lost their prognostic significance once the effect was corrected for perfusion variables. This association also emphasized the primacy of uterine over ovarian perfusion and the final assessment of these parameters after ovarian stimulation over their initial baseline values. The latter limits the clinical application of these parameters in pretreatment evaluation of the patients for their prognostic potential to tailor their ovarian stimulation but can still be valuable in management of ET in terms of number of embryos (Table 5.32-5.35).

Using a more focused list of independent variables including only VEGF and VEGFR concentrations and Doppler indices, logistic regression analyses showed that only pre- and post-treatment serum VEGF were the independent prognosticators of pregnancy. This may reflect the foremost role of angiogenetic stimulation and tissue perfusion in the overall down-stream process of conception.

7 Frozen-Thawed Embryo Replacement Cycles: Results

7.1 Clinical and Embryological Variables of the FTER and their Primary Fresh IVF Treatment Cycles

From the total study population, 21.9% of the couples were randomised to either the NC or DRRC groups. The remaining 78.1% of the couples expressed a strong preference for one mode of treatment and were allocated to either the NC or DRRC groups according to their preference. In the NC FTER group 18% of cycles were randomised and in the DRRC FTER group 26.5% were randomised. Of all the FTER cycles, 53% were in the NC group and 47% in the DRRC group.

Demographic characteristics of the couples and details of the primary fresh IVF treatment cycles relating to the index FTER cycles were summarized (Table 7.1, 7.2, *Table 7.1-7.16CD*). For all FTER cycles, the average number of embryos implanted per cycle was 0.3, the pregnancy rate 26.7% and on-going pregnancy 21% *(Table 7.17CD).*

Table 7.1: Patient Characteristics, Clinical and Embryological Variables of the NC and DRRC Groups in their Primary Fresh IVF Treatment Cycle

		Natural Cycle		DRRC	
		N	Mean ± SD	N	Mean ± SD
Female Age (year)		56	34.6 ± 5.0	49	33.4 ± 4.3
Duration of Subfertility (month)		56	49.0 ± 30.3	49	59.5 ± 38.8
Gravity		56	0.8 ± 1.1	49	0.7 ± 1.6
Parity		55	0.3 ± 0.6	47	0.3 ± 0.5
Duration of Menstrual Cycle (day)		56	28.5 ± 4.1	49	30.1 ± 2.6
Duration of Menstrual Bleeding (day)		56	4.9 ± 1.1	49	5.1 ± 1.1
BMI (kg/m^2)		55	24.1 ± 4.3	47	23.1 ± 3.3
Baseline FSH (IU/l)		51	7.5 ± 3.9	48	6.1 ± 1.8
Initial Gonadotrophin Dose (IU)		56	170 ± 45.3	49	155 ± 36.5
Duration of Gonadotrophin Stimulation (day)		56	12.0 ± 2.7	49	11.5 ± 2.5
Total Gonadotrophin Dose (IU)		56	2067 ± 800	49	1748 ± 618
E_2 Level after Down-Regulation (nmol/l)		56	0.08 ± 0.02	49	0.08 ± 0.0
E_2 Level before hCG Injection (nmol/l)		56	6.6 ± 3.6	49	8.4 ± 2.6
Number of Follicles measuring	< 9 mm	56	4.0 ± 6.6	49	5.8 ± 7.1
	10-15 mm	56	7.2 ± 4.8	49	8.8 ± 4.4
	16-17 mm	56	2.8 ± 1.6	49	3.2 ± 1.6
	18-20 mm	56	2.3 ± 1.2	49	2.8 ± 1.8
	21-25 mm	56	0.4 ± 0.7	49	0.5 ± 0.8
	26-30 mm	56	0.0 ± 0.0	49	0.04 ± 0.29
Number of Follicles Aspirated		56	15.4 ± 5.8	49	18.8 ± 5.7
Number of Oocytes Collected		56	14.1 ± 5.8	49	17.7 ± 6.1
Number of Oocytes Used		53	13.3 ± 5.5	49	17.3 ± 5.6
Number of 2-PN Embryo		55	9.9 ± 3.9	49	12.7 ± 4.0
Number of Embryos Transferred		56	1.7 ± 0.7	49	1.2 ± 0.9
Number of Embryos Cryopreserved		56	8.2 ± 7.6	49	9.9 ± 4.7
Number of Frozen after Cell Division		48	5.4 ± 2.6	44	6.9 ± 3.1
Number of Frozen at Pronuclear Stage		14	8.5 ± 4.3	21	8.8 ± 3.2
Grade of the First Embryo Transferred		48	1.1 ± 0.4	32	1.1 ± 0.4
Grade of the Second Embryo Transferred		48	1.3 ± 0.6	31	1.2 ± 0.5
Grade of the Third Embryo Transferred		4	1.5 ± 0.5	0	
Embryo Transfer Time (sec)		48	46.8 ± 46.6	32	53.5 ± 64.8
Number of Embryos Implanted in Fresh Cycle		53	0.3 ± 0.5	45	0.1 ± 0.3
Number of Embryos Implanted in FTER Cycle		55	0.3 ± 0.6	47	0.3 ± 0.5

N: Number of subjects whose data were available for each variable
Percentages represented as decimal of 1.0 ± SD

Table 7.2: Outcome of the Fresh IVF Cycle in the NC and DRRC Groups

	Outcome of Fresh Cycle	N	Percent
Natural Cycle	Pregnant Ongoing	13	23.2
	Pregnant Miscarriage	5	8.9
	Pregnant Biochemical	3	5.4
	Not pregnant	35	62.5
DRRC	Pregnant Ongoing	5	10.2
	Pregnant Miscarriage	2	4.1
	Pregnant Biochemical	4	8.2
	Not pregnant	38	77.6

In the NC FTER group, the median day of LH surge was cycle day 14 (9-23). On the day that onset of the surge was first detected, the average LH level was 35.1 IU/l (7.8 - 100) with E_2 concentration of 1 nmol/l (0.29-1.88). After detection of the onset of the surge, LH levels continued to increase to a peak of 45.2 nmol/l (13.8-124.4). ET was scheduled for day 4 following detection of the onset of LH surge (cycle days 3-6). A median of 3 embryos were thawed (1-7), 2 survived (1-5), and 2 were transferred (1-3). The three embryos selected for transfer were Grade 1, 2, and 3 respectively. After the index study cycle a median of 2 embryos remained for future transfer (0-16). The average mid-luteal P_4 concentration was 44 nmol/l. Of the NC, 71% graded the ET procedure 'easy' and 1.8% 'difficult'. The pregnancy and ongoing pregnancy rates were 25% and 19.6% respectively. *(Table 7.18, 7.20, 7.21CD).*

In the DRRC FTER group, the average E_2 and P_4 levels after down-regulation were 0.1 nmol/l (0.07-0.76) and 1.6 nmol/l (0.06-6.6) respectively. A median of 3 embryos were thawed (2-13), 2 survived (1-11), and 2 were transferred (1-3). The three embryos selected for transfer were Grade 1, 2, and 3 respectively. Following the index study cycle a median of 5 embryos remained for future transfer (0-16). Of the DRRC, 67% graded the ET procedure 'easy' and 10.2% 'difficult. The pregnancy and ongoing pregnancy rates were 28.6% and 22.4% respectively.' *(Table 7.19CD).*

7.1.1 Correlation Between Clinical and Embryological Variables of the NC

Correlations observed among the clinical and embryological variables of the NCs were summarized (Table 7.3). Positive correlation between the variables was given as '↑', negative correlation as '↓', and no correlation as "↔". Correlations with a p value of <0.05 were marked with '*', and those with a p value of 0.05-0.09 with '**'. Numerical values of the correlation coefficients and corresponding p values were given on the CD Rom *(Table 7.22-7.24CD).*

Table 7.3: Correlations between Clinical and Embryological Variables in the NC

	LH Surge				**Number of Embryos**			
	Day	**E_2 Level**	**LH Level**	**Maximum LH Level**	**Thawed**	**Survived**	**Left**	**Transferred**
Day of LH Surge	↔	↔	↔	↔	↔	↔	↑*	↔
E_2 Level at LH Surge	↔	↔	↔	↔	↔	↔	↔	↑**
LH Level at LH Surge	↔	↔	↔	↑*	↔	↔	↔	↔
Maximum LH Level	↔	↔	↑*	↔	↔	↔	↔	↔
ET Day	↑*	↔	↓*	↔	↔	↔	↔	↔
Embryos Thawed	↔	↔	↔	↔	↔	↑*	↔	↑*
Embryos Survived	↔	↔	↔	↔	↑*	↔	↔	↑*
Embryos Left	↑*	↔	↔	↔	↔	↔	↔	↔
Embryos Transferred	↔	↑**	↔	↔	↑*	↑*	↔	↔
Grade of the 1st Embryo	↔	↓*	↔	↔	↑*	↔	↓**	↔
Grade of the 2nd Embryo	↔	↔	↔	↔	↑**	↔	↔	↔
Grade of the 3rd Embryo	↔	↓*	↔	↔	↔	↔	↔	↔
Luteal Phase P_4 Level	↔	↔	↔	↔	↔	↔	↔	↔

↑: Positive correlation between the variables, ↓: negative correlation, ↔ : no correlation, * : p:<0.05, ** : p:0.05-0.09.

7.2 Clinical and Embryological Variables of the Primary Fresh IVF Treatment Cycle in Predicting the outcome of Subsequent FTER in the Whole Study Population

The data were evaluated to assess the prognostic significance of the clinical and embryological variables of the primary fresh IVF treatment cycle, to predict the outcome of the FTER cycle in terms of ongoing pregnancy *(Table 7.25-7.38CD)*.

In the primary fresh IVF treatment cycle, the parameters that showed significantly different distribution between the pregnant (ongoing) and non-pregnant groups of the subsequent FETR cycles respectively (p:<0.05) were female height (1.66 vs 1.62cm), number of developing follicles particularly between 10-15mm (10.5 vs 7.2), number of follicles aspirated (20.8 vs 16), number of oocytes collected (19 vs 14), number of oocytes used for fertilisation (18 vs 14), number of 2-PN embryos (12.9 vs 10.8), number of embryos transferred (0.8 vs 1.7) and number of embryos cryopreserved (11 vs 8). Being randomised or allocated on personal choice to the NC or DRRC groups did not affect the outcome of FETR.

The pregnant and non-pregnant groups in the FTER cycle showed no difference when the following variables were compared in the primary fresh IVF treatment cycle: female age, duration of subfertility, past obstetric history, duration of menstrual cycle, female weight and BMI, early follicular phase FSH, initial daily gonadotrophin dose and total gonadotrophin dose, duration of ovarian stimulation, E_2 levels after down-regulation and before hCG injection, sperm concentrations before and after preparation and grade of the embryos transferred. Primary and secondary male or couple subfertility did not affect the outcome of the FTER cycle. However, in contrast to general expectation, women with secondary subfertility were less likely to achieve ongoing pregnancy in an FTER cycle (p: 0.01). The aetiology of subfertility and the female life-style in terms of smoking and alcohol consumption did not affect the treatment outcome of FTER. When the pregnant and non-pregnant groups in the FTER cycles were compared, there were no differences in the use of mid-luteal or early follicular protocols, type of GnRHa, type of gonadotrophin, type of

fertilization (IVF vs ICSI) and the use of husband or donor sperm in the primary fresh IVF treatment cycles. The outcome of the FTER cycles was not influenced by the degree of technical difficulty of the ET in either the primary fresh IVF treatment cycle or FTER cycles for both NCs and DRRCs *(Table 7.26-7.38CD).* The probability of pregnancy in FTER was higher with pronuclear stage cryopreservation when compared with cleavage stage (p: 0.05).

The outcome of the fresh IVF treatment in terms of ongoing pregnancy, biochemical pregnancy, miscarriage, or no-pregnancy did not affect the outcome of the subsequent FTER cycle. However, it is of note that of the women with biochemical pregnancy or miscarriage in the fresh IVF treatment cycle, none achieved ongoing pregnancy in the FTER cycle (Table 7.4). The outcome of the primary fresh IVF treatment cycle did not affect the type of FTER cycles to be used for the subsequent index study cycle and had no influence on whether couples agreed to randomisation or had a strong preference for a particular type of FTER (NC or DRRC) (Table 7.5).

Table 7.4: Association between the Outcome of Fresh IVF Cycle and FTER in the Whole Study Population

			Outcome of Fresh Treatment Cycle				
			Pregnant Ongoing	**Miscarriage**	**Biochemical**	**Not Pregnant**	**p**
Outcome of FTER	Pregnant	Yes	3	1	0	24	NS
		No	15	6	7	49	
	Ongoing Pregnant	Yes	2	0	0	20	NS
		No	16	7	7	53	

Table 7.5: Association between the Outcome of Fresh Cycle and Type of FTER and Randomization Status in the Whole Study Population

	Outcome of Fresh Treatment Cycle				
	Pregnant Ongoing	**Miscarriage**	**Biochemical**	**Not pregnant**	**p**
Natural Cycle	13	5	3	35	NS
DRRC	5	2	4	38	
Randomized	3	1		19	NS
Non-Randomized	15	6	7	54	

7.3 Clinical and Embryological Variables of the NC in Predicting the Outcome of FTER

When clinical and embryological variables of the NC were evaluated for clinical pregnancy between the pregnant and non-pregnant groups respectively, the number of remaining cryopreserved embryos after FTER (5.7 vs 2.1) and mid-luteal P_4 levels (52 vs 40) were found to be significantly different (p:<0.05) *(Table 7.39, 7.40CD).*

Clinical and embryological variables were evaluated for their prognostic significance to predict ongoing pregnancy after NC FTER. Between the two outcome groups respectively, there was no difference for the day of LH surge (15 vs 14), LH (33 vs 35 IU/l) and E_2 (1 vs 1 nmol/l) concentrations on the day of LH surge, maximum LH concentration (peak level of surge) (47 vs 44 IU/l), day of ET after LH surge (4 vs 5), number of embryos thawed (3.7 vs 3.1), number of embryos that survived thawing (2.5 vs 2.3), number of remaining cryopreserved embryos (6.5 vs 2.1), number of embryos transferred (2 vs 2), grade of the first (1 vs 1.44), second and third embryos transferred, duration of ET, technical difficulty of ET and mid-luteal P_4 level (47.7 vs 43.3 nmol/l) *(Table 7.41, 7.42CD).*

7.4 Clinical and Embryological Variables of the DRRC in Predicting the Outcome of FTER

When clinical and embryological variables in the DRRC group were evaluated for clinical pregnancy, the grades of the first (1 vs 1.4) and second (1.5 vs 2) embryos selected for transfer and duration of the ET procedure (92 vs 34 sec) were found to be significantly different (p:<0.05) between the pregnancy and non-pregnancy outcomes *(Table 7.43, 7.44CD).*

Clinical and embryological parameters were evaluated for their prognostic significance to predict ongoing pregnancy after DRRC FTER. There was no difference between the two outcome groups for E_2 (0.12 vs 0.15 nmol/l) and P_4 (1.98 vs 1.49 nmol/l) concentration after down-regulation, number of embryos thawed (3.2 vs 3.6), number of embryos that survived thawing (2.6 vs 2.6), number of remaining cryopreserved embryos (7.3 vs 5), grade of the first (1 vs

1.36), second (1.6 vs 2), and third embryos transferred, number of embryos transferred (2 vs 2), and duration and technical difficulty of the ET procedure *(Table 7.45, 7.46CD).*

7.5 Comparison of the Clinical and Embryological Variables in the Primary Fresh IVF Treatment Cycles of the NC vs DRRC Groups

Clinical and embryological variables of the primary fresh IVF treatment cycles were compared, to assess whether they differed between the related NC and DRRC groups *(Table 7.47CD).*

The following variables of the fresh treatment cycle differed significantly between the NC and DRRC groups, respectively (p:<0.05): early follicular phase FSH levels (7.5 vs 6.1 IU/l), initial daily gonadotrophin dose (170 vs 155 IU) and total gonadotrophin dose (2067 vs 1748 IU), E_2 level on the day of hCG injection (6.6 vs 8.4 nmol/l), number of developing follicles measuring 10-15mm (7.2 vs 8.8), total number of follicles aspirated (15 vs 18), total number of oocytes collected (14 vs 17), total number of oocytes used for fertilisation (13 vs 17), number of 2-PN embryos created (9.9 vs 12.7), number of embryos transferred (1.79 vs 1.29), number of embryos cryopreserved (8.2 vs 9.9), number of embryos cryopreserved after cell division (5.4 vs 6.9), and number of embryos implanted per fresh cycle initiated (0.3 vs 0.1).

For women in the DRRC group the length of the menstrual cycle was 1.5 days longer than for women in the NC group (28.5 vs 30 days; p: <0.05). There was no difference between the NC and DRRC groups for female age (34 vs 33 years), duration of subfertility (49 vs 59 months), past obstetric history, and BMI *(Table 7.47CD).* In both the NC and DRRC groups, the distribution of primary and secondary male and female subfertility was similar, but there were more couples with secondary subfertility in the NC group (p:0.01). No difference was noted between the NC and DRRC groups for aetiology of subfertility. Female life-style, in terms of smoking and alcohol consumption was similar in both groups.

Type of fertilization (IVF or ICSI) used in the fresh IVF cycles was similar in both groups. The distribution of divided and pronuclear stage embryos was equal in the NC and DRRC groups *(Table 7.48-7.58CD).* The majority of the fresh ET procedures were graded 'easy', but the women in the NC group appeared to have easier fresh ETs than women in the DRRC group (p: 0.04). In the DRRC group 10.2% of women experienced a difficult FTER and in the NC group this was 1.8% (p: 0.17) *(Table 7.59, 7.60CD).* There was no difference in the grade of the embryos transferred in the fresh cycle *(Table 7.47CD).*

Although, differences between the NC and DRRC groups in the outcome of their primary fresh IVF treatment cycles were not statistically significant, it may be of particular interest to review the absolute values. From the primary fresh IVF treatment cycle 72% of women with ongoing pregnancy subsequently underwent a NC and 27.8% DRRC. Where conception was not achieved in the primary fresh IVF treatment cycle, 47.9% of the women were in the NC group and 52.1% in the DRRC group (Table 7.5). When we reviewed the outcome of the related non-study FTER cycles using embryos from the cohort created in the fresh IVF cycle, but not transferred in the index study FTER cycle, no difference was observed in the pregnancy rates of the two groups (Table 7.6).

Table 7.6: Outcome of the Other Related FTER for NC vs DRRC Groups

		Type OF FTER		P
		Natural Cycle	DRRC	
Pregnancy with other FTER cycles	Pregnant	2	6	NS
	Not Pregnant	19	15	

In the NC and DRRC groups, pregnancy and ongoing pregnancy rates of FTER were similar (Table 7.7) as was the quality of transferred embryos in FTER *(Table 7.77CD).*

Table 7.7: Outcome of the Study FTER for NC vs DRRC Cycles

		Natural Cycle	DRRC	P
Pregnant	Yes	14	14	NS
	No	42	35	
Ongoing Pregnancy	Yes	11	11	NS
	No	45	38	

Ten women in the NC group and 13 in the DRRC group were randomised and the remaining women allocated to either group according to their preference. Couples who were randomized or allocated to their treatment cycles based on personal preference were compared, to assess whether there were any differences which may affect the homogeneity of the study groups and so confound the comparative statistics *(Table 7.61CD).*

Only duration of menstrual cycle differed between the randomized and allocated groups respectively (28 vs 29 days; p:<0.01). Although the difference was statistically significant, the variation of one day in a range of 26 to 36 days is less likely to be clinically relevant. Female age and BMI, early follicular phase FSH level, duration of subfertility, past obstetric history, sperm parameters, and clinical and embryological parameters of ovarian stimulation were similar in both groups *(Table 7.61CD).*

There was no difference between the randomised and allocated groups in distribution of female, male and couple subfertility, aetiology of subfertility, female smoking and alcohol consumption, use of the day 2 or day 21 protocol, type of GnRHa down-regulation in the primary fresh IVF treatment cycle, type of gonadotrophin used for ovarian stimulation, number of divided or pronuclear stage embryos, difficulty of ET in fresh and FTER cycles, type of FTER cycle (NC or DRRC) or outcome of the fresh ET and FTER cycle (Table 7.8, 7.9, *Table 7.62-7.76CD).*

Table 7.8: Association between the State of Randomization and the Outcome of the FTER Cycles

			Allocation		P
			Randomized	Non-randomized	
Outcome of FTER	Pregnant	Yes	5	23	NS
		No	18	59	
	Ongoing Pregnancy	Yes	5	17	NS
		No	18	65	

Table 7.9: Randomization for the Type of FTER Groups

	Allocation		P
	Randomized	Non-randomized	
Natural Cycle	10	46	NS
DRRC	13	36	

7.6 Factors Linked to Embryo Survival Rate

In the NC group the embryo survival rate (ESR) did not correlate with any of the patient characteristics, clinical and embryological variables of the primary fresh IVF treatment and subsequent FTER cycles, including the number of embryos implanted after FTER, serum VEGF and VEGFR levels on the day of ET, and grade of embryos selected for fresh ET *(Table 7.78-7.83, 7.110CD).*

In the DRRC group, the embryo survival rate did not correlate with any of the clinical and embryological parameters, with the exception of positive correlation with the number of developing follicles at 16-17mm (p: 0.01), total number of follicles aspirated (p: 0.01), number of oocytes collected (p: <0.05), number of oocytes used for fertilisation (p: <0.05) and number of 3-PN embryos (p: <0.01) and negative correlation with the number of embryos transferred during the fresh IVF cycle (p: 0.01) *(Table 7.78-7.83CD).*

In both the NC and DRRC groups, there was an association between higher ESR and a diagnosis of secondary female subfertility but this approached statistical significance only in the DRRC group (p: 0.05). Male and couple subfertility and type of subfertility did not correlate with ESR. Female smoking and alcohol consumption did not affect ESR. A day 2 or day 21 start for down-regulation and type of fertilisation (IVF or ICSI) did not affect ESR (*Table 7.84-7.103CD).*

The outcome of the fresh IVF cycle did not affect the survival rate of cryopreserved embryos in a subsequent study FTER cycle *(Table 7.104, 7.105CD);* and in both the NC and DRRC groups, the embryo survival rate did not affect the pregnancy and ongoing pregnancy rates of the FTER cycles *(Table 7.106-7.109CD).* Although quality of the embryos transferred in the fresh

IVF cycle did not affect the ESR in either NCs or DRRCs, ESR was associated with the quality of the best embryo transferred in both FTER groups *(Table 7.110CD).*

In the DRRC group, ESR was significantly higher if embryos were cryopreserved at the pronuclear stage rather than after cell division (p: <0.05) (Table 7.10, 7.11).

Table 7.10: Association between Embryo Survival Rate and Stage of Embryo Development in NC and DRRC

Embryo Survival Rate (%)		N	Mean ± SD	p
Natural Cycle	Divided	38	0.7 ± 0.2	NS
	Pro Nuclear	4	0.9 ± 0.1	
	Divided and Pro Nuclear	10	0.7 ± 0.3	
DRRC	Divided	28	0.7 ± 0.2	0.01
	Pro Nuclear	5	1.0 ± 0.0	
	Divided and Pro Nuclear	16	0.9 ± 0.1	

Percentages represented as decimal of 1.0 ± SD

Table 7.11: Association between Embryo Survival Rate and Stage of Embryo Development in DRRC (Bonferroni Multiple Comparisons)

			p
DRRC Embryo Survival Rate	Divided vs	Pro Nuclear	<0.05
		Divided and Pro Nuclear	<0.05
	Pro Nuclear vs	Divided	<0.05
		Divided and Pro Nuclear	NS

7.7 Cleavage Stage vs Pro-nuclear Stage Embryo at the time of Cryo-preservation

Embryos cryopreserved at the pronuclear stage were compared with embryos cryopreserved after cleavage stage. In the DRRC group the survival rate was significantly higher for pronuclear-stage embryos (100% vs 72%) and this trend was evident in the NC group but not at a significant level (93% vs 78%). Although differences in clinical pregnancy rates were not statistically significant (pronuclear stage 44.4% > cleavage stage 19.7%), ongoing pregnancy rates (pronuclear stage 33.3% > cleavage stage13.6%; p:0.05) and number of embryos implanted (pronuclear stage 0.55 > cleavage stage 0.18 p:<0.05) showed a clear distinction. This effect was probably due to the survival ability of

the embryos, as there was no difference in the embryo quality between PN and cleavage stage *(Table 7.111-7.113CD).* When we separately evaluated the NC and DRRC groups, there was an evident trend of embryos cryopreserved at the pronuclear stage having higher survival and pregnancy outcome rates than embryos divided at the time of cryopreservation. Because, the number of cycles was reduced in this subgroup analysis, statistical significance appeared to be lower *(Table 7.114-7.119CD).*

7.8 VEGF, VEGFR, VEGF:VEGFR in Predicting the Treatment Outcome in the Whole Study Population

VEGF and VEGFR concentrations were measured in 61 serum samples obtained on the day of FTER (Table 7.12). In pregnant and non-pregnant women, on the day of FTER the difference in serum VEGF and VEGFR concentrations was not statistically significant. This was also the case in women with ongoing pregnancy and those with no-ongoing pregnancy (Table 7.13).

Despite not reaching statistical significance, higher VEGF and lower VEGFR levels were evident in women who achieved pregnancy after FTER and this distinction became more marked in those with ongoing pregnancy (Table 7.13).

Table 7.12: VEGF, VEGFR Levels

	N	Mean ± SD
VEGF (pg/ml)	61	306.5 ± 196.0
VEGFR (pg/ml)	61	37.6 ± 18.7

Table 7.13: VEGF, VEGFR levels for Clinical and Ongoing Pregnancy in the Whole Study Population

	Clinical Pregnancy	N	Mean ± SD	p
VEGF (pg/ml)	Yes	16	331.3 ± 231.0	NS
	No	45	297.7 ± 184.0	
VEGFR (pg/ml)	Yes	16	34.3 ± 11.2	NS
	No	45	38.7 ± 20.7	
	Ongoing Pregnancy			
VEGF (pg/ml)	Yes	13	346.2 ± 248.3	NS
	No	48	295.7 ± 180.9	
VEGFR (pg/ml)	Yes	13	34.7 ± 12.5	NS
	No	48	38.3 ± 20.1	

Differences in serum VEGF and VEGFR levels did not reach statistical significance in women with primary or secondary subfertility, women who are smokers or non-smokers and women who do or do not consume alcohol *(Table 7.120-7.122CD).*

VEGF levels did not differ in different aetiologies of subfertility, unlike VEGFR levels. Levels of VEGFR in unexplained subfertility (25.3 pg/ml) were significantly lower than in tubal factor subfertility (43.7 pg/ml) (p: <0.05). Lower levels of VEGFR in endometriosis (29.2 pg/ml) and higher levels in male factor subfertility (39.2 pg/ml) were not significantly different *(Table 7.123, 7.124CD).*

The ratio of VEGF to its receptor did not improve the discriminative power of the individual parameters in the prediction of pregnancy following FTER. However, a higher ratio was evident in pregnant cycles (Table 7.14, 7.15).

Table 7.14: VEGF:VEGFR Ratio

	N	Mean ± SD
VEGF:VEGFR	61	10.3 ± 8.9

Table 7.15: VEGF:VEGFR Ratio for Clinical Pregnancy

	N	Mean ± SD	p
Pregnant	16	11.1 ± 9.7	NS
Not Pregnant	45	10.0 ± 8.6	

7.8.1 VEGF, VEGFR, VEGF:VEGFR in Predicting the Treatment Outcome in the NC vs DRRC Groups

Serum VEGF, VEGFR, and VEGF:VEGFR levels were not significantly different between pregnant and non-pregnant women in both the NC and DRRC groups. However, the NC group had lower VEGF and VEGFR levels in pregnancy cycles than in non-pregnancy cycles (NS). The DRRC group had higher VEGF and lower VEGFR levels in pregnancy cycles than in non-pregnancy cycles (NS). (Table 7.17, 7.18).

VEGFR levels were significantly lower in DRRCs when compared with NCs (Table 7.16). This difference between NCs and DRRCs was mainly observed in

the pregnant women, as VEGFR in non-pregnant women was similar in NCs and DRRCs (Table 7.19-7.21).

VEGF levels were similar in both NCs and DRRCs. However, the absolute VEGF levels were higher in the DRRC group than in the NC group with pregnancy, but the reverse was true if pregnancy was not achieved (NS) (Table 7.19-7.21).

In both the NC and DRRC groups, the diagnosis of subfertility did not affect VEGF levels at a statistically significant level. In the NC group the differences in VEGFR levels were statistically significant (p:0.01) and the levels in unexplained subfertility were significantly lower than in tubal factor subfertility *(Table 7.128CD).*

Table 7.16: VEGF and VEGFR levels for NC vs DRRC

		N	Mean ± SD	p
VEGF (pg/ml)	Natural Cycle	35	301.1 ± 176.5	NS
	DRRC	26	313.7 ± 223.0	
VEGFR (pg/ml)	Natural Cycle	35	40.5 ± 18.6	<0.05
	DRRC	26	33.6 ± 18.5	

Table 7.17: VEGF, VEGFR, VEGF:VEGFR for Clinical Pregnancy in NC and DRRC

Natural Cycle				
	Pregnant	**N**	**Mean ± SD**	**p**
VEGF: VEGFR	Yes	10	7.5 ± 4.1	NS
	No	25	9.4 ± 7.4	
VEGF (pg/ml)	Yes	10	287.4 ± 162.3	NS
	No	25	306.7 ± 184.8	
VEGFR (pg/ml)	Yes	10	39.2 ± 10.7	NS
	No	25	41.0 ± 21.2	

DRRC				
	Pregnant	**N**	**Mean ± SD**	**p**
VEGF: VEGFR	Yes	6	17.1 ± 13.6	NS
	No	20	10.6 ± 10.1	
VEGF (pg/ml)	Yes	6	404.6 ± 320.0	NS
	No	20	286.4 ± 187.2	
VEGFR (pg/ml)	Yes	6	26.1 ± 6.7	NS
	No	20	35.9 ± 20.3	

Table 7.18: VEGF, VEGFR for Ongoing Pregnancy in NC and DRRC

Natural Cycle				
	Ongoing Pregnancy	**N**	**Mean ± SD**	**p**
VEGF (pg/ml)	Yes	8	289.8 ± 165.5	NS
	No	27	304.5 ± 182.5	
VEGFR (pg/ml)	Yes	8	40.9 ± 11.4	NS
	No	27	40.3 ± 20.5	

DRRC				
	Ongoing Pregnancy	**N**	**Mean ± SD**	**p**
VEGF (pg/ml)	Yes	5	436.5 ± 347.0	NS
	No	21	284.4 ± 182.7	
VEGFR (pg/ml)	Yes	5	24.8 ± 6.7	NS
	No	21	35.7 ± 19.8	

Table 7.19: VEGF, VEGFR, VEGF:VEGFR Levels in the Pregnant Group of NC vs DRRC

Pregnant Women				
		N	**Mean ± SD**	**p**
VEGF: VEGFR	Natural Cycle	10	7.5 ± 4.1	0.05
	DRRC	6	17.1 ± 13.6	
VEGF (pg/ml)	Natural Cycle	10	287.4 ± 162.3	NS
	DRRC	6	404.6 ± 320.0	
VEGFR (pg/ml)	Natural Cycle	10	39.2 ± 10.7	<0.05
	DRRC	6	26.1 ± 6.7	

Table 7.20: VEGF, VEGFR, VEGF:VEGFR Levels in the Non-pregnant Group of NC vs DRRC

Non-pregnant Women				
		N	**Mean ± SD**	**p**
VEGF: VEGFR	Natural Cycle	25	9.4 ± 7.4	NS
	DRRC	20	10.6 ± 10.1	
VEGF (pg/ml)	Natural Cycle	25	306.7 ± 184.8	NS
	DRRC	20	286.4 ± 187.2	
VEGFR (pg/ml)	Natural Cycle	25	41.0 ± 21.2	NS
	DRRC	20	35.9 ± 20.3	

Table 7.21 : Summary of Associations between VEGF-VEGFR and Treatment Outcome

	Whole Population	**DRRC**	**NC**
VEGF	Higher in pregnant	Higher in pregnant Higher than NC in pregnant Lower than NC in non-pregnant	Lower in pregnant
VEGFR	Lower in pregnant	Lower in pregnant Lower than NC in pregnant ($p<0.05$) Lower than NC in non-pregnant	Lower in pregnant

7.9 Endometrial Echo-pattern of FTER Cycles

Three different endometrial patterns were recognised on the day of FTER: w, w-b-w and w-b-ml-b-w, in which 'w' represents the hyperechogenic zone, 'b' the hypoechogenic zone, and 'ml' the midline echo.

In the whole study population, female age, height, weight or BMI, duration of subfertility, gravidity, parity, duration of menstrual cycle, and early follicular phase FSH did not differ significantly between the endometrial patterns of w, w-b-w and w-b-ml-b-w *(Table 7.139CD).*

In the NC FTER group, the endometrial patterns w, w-b-w, and w-b-ml-b-w presented different levels of receptivity, as assessed by the average number of embryos implanted per cycle (p:0.05), parity (p:0.08), and mid-luteal P_4 concentrations (p:0.09) (Table 7.22, *Table 7.140CD).*

In the DRRC group, the endometrial patterns w, w-b-w, and w-b-ml-b-w did not differ in association with any of these parameters.

In the NC FTER group, the w-b-w pattern was associated with the highest implantation rate per cycle (p:0.05), and a trend for the highest number of previous pregnancies beyond viability, the highest serum VEGF and lowest VEGFR level on the day of assessment, the highest LH level on the day of surge, the highest maximum LH level during the surge, and the highest mid-luteal P_4 (NS). In these associations the w pattern appeared to be placed between the w-b-w and w-b-ml-b-w patterns. This trend was not evident in the DRRC group (Table 7.22, *Table 7.140CD).*

The endometrial pattern did not differ with the type of female subfertility, diagnosis of subfertility, smoking, or alcohol consumption and this was valid for the NC and DRRC groups *(Table 7.141-7.144CD).* The endometrial pattern did not differ between pregnant and non-pregnant women (Table 7.23).

Table 7.22: Clinical and Embryological Variables for Different Endometrial Echo-patterns

Natural Cycle				
	Endometrial Pattern	**N**	**Mean ± SD**	**p**
VEGF (pg/ml)	w.b.ml.b.w	12	278.4 ± 117.9	NS
	w.b.w	6	336.8 ± 212.8	
	w	17	296.3 ± 211.5	
VEGF Receptor (pg/ml)	w.b.ml.b.w	12	41.5 ± 11.5	NS
	w.b.w	6	36.2 ± 13.9	
	w	17	40.5 ± 24.6	
Number of Implanted Embryos FTER	w.b.ml.b.w	21	0.1 ± 0.4	0.05
	w.b.w	11	0.7 ± 0.9	
	w	19	0.2 ± 0.5	
LH Level on the Day of LH Surge (IU/l)	w.b.ml.b.w	21	35.4 ± 23.5	NS
	w.b.w	11	38.3 ± 23.3	
	w	20	34.7 ± 18.9	
Maximum LH Level (IU/l)	w.b.ml.b.w	21	45.1 ± 20.3	NS
	w.b.w	11	56.8 ± 30.9	
	w	20	39.8 ± 19.8	
Luteal P_4 Level (nmol/l)	w.b.ml.b.w	14	38.9 ± 15.7	NS
	w.b.w	10	54.5 ± 15.0	
	w	20	44.9 ± 18.6	
Parity	w.b.ml.b.w	21	0.1 ± 0.4	NS
	w.b.w	11	0.7 ± 0.9	
	w	20	0.4 ± 0.6	

Table 7.23: Associations between Endometrial Echo-pattern and Treatment Outcome in FTER

		Endometrial Pattern			**p**
		w-b-ml-b-w	**w-b-w**	**w**	
Pregnancy	Yes	10	6	6	NS
	No	35	9	26	
Ongoing Pregnancy	Yes	8	5	5	NS
	No	37	10	27	

7.10 Endometrial Thickness of FTER Cycles

In the FTER group, regardless of type of endometrial preparation, on the day of ET the mean endometrial thickness was 9.5mm (3.7-15mm) (Table 7.24, 7.25).

Table 7.24: Endometrial Thickness in the Whole Study Population

Endometrial Thickness	
Mean ± SD	9.5 ± 2.7 mm
Minimum	3.7 mm
Maximum	15.0 mm

Table 7.25: Frequency Distribution of Endometrial Thickness in the Whole Study Population

Endometrial Thickness	**Cumulative Percent**
5 mm and less	4.3
5 to 6.9 mm	20.7
7 to 8.9 mm	39.1
9 to 10.9 mm	77.2
11 to 15 mm	100.0

In the NC and DRRC groups the mean endometrial thickness was 9.5 mm and 9.6 mm respectively. Although the mean values were almost the same, the distribution around this mean differed in both groups. In the NC group 7.7% had an endometrial thickness of ≤ 5 mm and in the DRRC group this was only 2.5%. Similarly, in the NC group 23% had an endometrial thickness of ≤ 7 mm and in the DRRC group this was only 17%. From this measurement of thickness upwards the distribution between the two groups is similar. This indicates that in the DRRC group, HRT could possibly correct the thin endometrium of some women who would otherwise have developed a thinner endometrium if they had had an NC (Table 7.26, 7.27).

Table 7.26: Endometrial Thickness for NC and DRRC

	N	**Mean**	**Min**	**Max**	**p**
Natural Cycle	52	9.5 ± 2.8 mm	3.7 mm	15.0 mm	NS
DRRC	40	9.6 ± 2.5 mm	5.3 mm	15.0 mm	

Table 7.27: Frequency Distribution of Endometrial Thickness in the NC and DRRC

	Endometrial Thickness	Cumulative Percent
Natural Cycle	5 mm and less	7.7
	5 to 6.9 mm	23.1
	7 to 8.9 mm	44.2
	9 to 10.9 mm	76.9
	11 to 15 mm	100.0
DRRC	5 mm and less	2.5
	5 to 6.9 mm	17.5
	7 to 8.9 mm	40.0
	9 to 10.9 mm	77.5
	11 to 15 mm	100.0

In the whole study population, endometrial thickness positively correlated with the duration of subfertility (p: 0.03), weight (p: 0.02), and BMI (p: 0.08) of the patient. No correlation was observed with female age, past obstetric history, duration of menstrual cycle and bleeding, and early follicular phase FSH levels *(Table 7.145, 7.146CD).*

In the NC group, endometrial thickness correlated negatively with the day of LH surge (p: 0.03), with the LH level on the day of LH surge (p: 0.08), and with the maximum LH level during the surge (p: 0.01); and positively with the E_2 level on the day of LH surge (p: 0.03). In the DRRC group, endometrial thickness correlated negatively with VEGFR levels (p: 0.01), and positively with the duration of subfertility (p: 0.04) and with parity (p: 0.04) *(Table 7.147-7.150CD).* In both the NC and DRRC groups, no correlation was observed between endometrial thickness and the average number of embryos implanted per cycle (*Table 151CD*). The endometrial thickness did not differ with the type of female subfertility, diagnosis of subfertility, smoking, or alcohol consumption. This was valid for the NC and DRRC groups *(Table 7.152-7.155CD)*

7.10.1 Endometrial Thickness in Different Echo-patterns

Endometrial thickness did not differ significantly with different endometrial patterns and mean values of endometrial thickness were >9mm in all patterns. This observation was also valid for NCs and DRRCs separately (Table 7.28, *Table 7.156CD).*

In the last two analyses 'VEGFR', 'female weight', 'day of LH surge' and 'maximum LH level during the surge' were used to build the model by linear regression analysis using the forced entry method to assess the overall performance of all four variables and then the forward conditional entry method to assess the performance of the best predictor. Both revealed the maximum LH level as the only independent predictor with statistical significance at 5%. The final model with maximum LH level has the p value of 0.002 and explained 22% of the variation *(Table 7.164, 7.165CD).*

7.10.3 Prognostic Significance of Endometrial Thickness for Pregnancy in NCs and DRRCs

In both the NC and DRRC FTER groups, the mean value of endometrial thickness did not differ between the pregnant and non-pregnant groups *(Table 7.166, 7.167CD).* While the thinnest endometrium for pregnancy was 3.8 mm in NC, this figure was 7 mm in DRRC. Half of the clinical pregnancies in each group occurred in endometrial thickness of >9.6mm (Table 7.29).

In the NC group the thinnest endometrium for ongoing pregnancy was 4.8 mm and in the DRC group 7mm. In both groups, half of the ongoing pregnancies were achieved with endometrial thickness of 10 mm to 15 mm *(Table 7.168CD).*

Table 7.29: Frequency Distribution of Endometrial Thickness for Clinical Pregnancy in NC and DRRC

		Endometrial Thickness (mm)											
	Pregnant	3.70	3.80	4.80	5.30	5.40	5.50	5.90	6.10	6.20	6.60	6.80	7.00
Natural Cycle	Yes		1	1							2	1	
	No	1					1	1	1		2		1
DRRC	Yes												2
	No				1	1			2	1			
		Endometrial Thickness (mm)											
	Pregnant	7.20	7.30	7.40	7.50	8.40	8.50	8.70	8.80	8.90	9.10	9.30	9.40
Natural Cycle	Yes						1					1	
	No	2			1			5	1		1		1
DRRC	Yes	1											1
	No		1	2		1			1	1	2		2
		Endometrial Thickness (mm)											
	Pregnant	9.50	9.60	9.70	9.80	9.90	10.00	10.50	11.00	12.00	13.00	14.00	15.00
Natural Cycle	Yes			1			1		3	1			1
	No	1		1		1	3	1	3	4	1	2	3
DRRC	Yes		1				1		1		1		
	No		1	1	1	1	3		2	3	2	1	2

The number of pregnant and non-pregnant women at different values of their endometrial thickness is given.

8 Frozen-Thawed Embryo Replacement Cycles: Discussion

A total of 105 women were recruited for the study. Of these, 23 women (21.9%) were randomised to either the NC or DRRC groups. The remaining 82 women (78.1%) were allocated to the treatment group for which they expressed a strong preference. Overall, randomisation was 18% in the NC group (n: 10) and 26.5% in the DRRC group (n: 13). Of all the FTER cycles, 53% had NCs and 47% had DRRCs. All the couples recruited for the study completed their FTER cycles, with no cancellations arising from cycle monitoring or frozen-thawed embryo failure to survive.

The available data from published studies suffer from the methodological problem of comparing the response of two unidentical groups to two different therapeutic regimens, when women with proven ovulation and regular cycles are primarily given natural cycles and women with anovulation or irregular cycles are given hormone replacement cycles. In the current study, these methodological problems were minimised and, unlike any of the published literature, the importance of tissue perfusion in terms of VEGF-VEGFR levels was considered and discussed. The main limitation of the current study was its limited power to fulfil the requirements of the power calculation. Because the majority of couples expressed a strong preference for either an NC or a DRRC, a preference arm was included in the study to maximise data collection. However, a post-hoc analysis revealed that the measured prognostic variables were similar for the randomised and allocated couples.

The discussion section begins by evaluating the predictive role of the fresh IVF cycles for the outcome of subsequent FTER cycles. The prognostic importance of the variables of the FTER cycles will be appraised and compared against the background of the primary fresh IVF treatment cycles. The second section evaluates the factors that may affect embryo survival during cryopreservation and thawing, and this will be followed by a brief overview of the prognostic significance of cleavage stage and pronuclear stage embryos. The final section presents an argument on VEGF-VEGFR interaction in FTER cycles followed by the evaluation of the endometrial echo-pattern and thickness.

8.1.1 Predictive Role of the Variables of the Fresh IVF Treatment Cycles for the Outcome of Subsequent FTER Cycles

To predict the pregnancy outcome of the subsequent FTER cycle, the clinical and embryological variables of the primary fresh IVF treatment cycles were evaluated.

Demographic features of the female partners did not affect the probability of ongoing pregnancy after FTER cycles. These included age, duration of subfertility, previous obstetric history, type and aetiology of subfertility, BMI, FSH levels, and lifestyle in terms of smoking and alcohol consumption. Sperm parameters and the technical difficulty in ET during the primary fresh IVF cycles were not prognostic for the FTER cycles *(Table 7.25-7.36CD).* These findings did not agree with those of Karlstrom et. al.[549] and Wang et. al.[484] who analysed 3570 FTER cycles in 1438 couples, to evaluate the clinical circumstances that influence the potential for embryo implantation, and report that female age <40 years and non-tubal factor subfertility are associated with a more favourable implantation rate.

The clinical and embryological parameters of a better ovarian response to stimulation during the fresh IVF cycle were associated with a higher probability of ongoing pregnancy in the subsequent FTER cycles. This association could be explained by the availability of a greater number of embryos for the subsequent FTER cycles increasing the probability of selection of better quality embryos for transfer, but it could also be due to an inherently better embryo cohort.

In the primary fresh IVF treatment cycle, the quality of the embryos transferred did not affect the outcome of the subsequent related FTER cycles *(Table 7.25CD).* This finding may provide evidence against the argument that the success rate of the FTER cycles was compromised because the best quality embryos were already selected for transfer in the primary fresh IVF treatment cycle.

The outcome of the primary fresh IVF treatment cycle in terms of ongoing pregnancy, miscarriage, biochemical pregnancy or no pregnancy did not influence the outcome of the related FTER cycle (Table 7.4). This finding was in contrast to those published by Karlstrom et. al.[549] and Wang et. al.[484], who report a more favourable implantation rate in FTER cycles if the previous fresh ET cycle was successful. However, it is of note that in the current study cohort, none of the women with a biochemical pregnancy or miscarriage in the fresh ET cycle achieved ongoing pregnancy in the subsequent FTER cycle. Furthermore, as only one FTER cycle was evaluated from a possible series using the embryos created in the primary fresh IVF treatment cycle, the apparent absence of association between the outcome of two treatment modalities may be due to under-representation of the FTER cycles.

To reconsolidate the findings that only the better ovarian response in the primary fresh IVF treatment cycle (but not its surrogate demographic variables, or quality of the transferred embryos, or the actual outcome) can predict the outcome of the subsequent FTER cycle, the argument is presented that it is the quality of the embryo cohort as a whole that determines the eventual success of the treatment, whether this is achieved at fresh ET or subsequent FTER. The probability of random events dictates equal and independent probability of the outcome, and this may be the simple reason why the observation that neither the quality of the two selected embryos nor the outcome of their implantation in the fresh IVF treatment can reliably predict the implantation potential of other embryos in the subsequent FTER cycles. However, the two treatment modalities are hardly independent from each other. The link between them is the mutual embryo source (despite one being fresh and the other frozen-thawed) and the attempted implantation in the same endometrium (probably with different levels of receptivity), with one being exposed to the high hormonal milieu of ovarian stimulation and the other to natural or hormone replacement levels.

No difference was observed in the outcome of the primary fresh IVF treatment cycle between couples randomised or allocated to the NC or DRRC groups (Table 7.5).

8.1.2 Clinical and Embryological Variables of the FTER Cycles

No correlation was found between the day of LH surge and the level of LH, or E_2 on the day of surge, or P_4 in the mid-luteal phase. Furthermore, E_2 levels on the day of surge did not correlate with P_4 levels in the mid-luteal phase. Therefore, it is the trend in, and not the absolute values of, E_2 or LH concentrations that should be considered during cycle monitoring for the prospective timing of ovulation and subsequent ET. Similarly, the absolute values of these two hormones had no association with the post-ovulatory P_4 rise and so could not be used to predict the likelihood of ovulation or the need for luteal support *(Table 7.22-7.24CD)*.

The E_2 level on the day of LH surge correlated positively with the quality of the transferred embryos. This association could reflect the link between the competency of folliculogenesis measured by the surrogate marker of E_2 concentration, and the quality of oocyte maturation. Despite the embryos being created in a different ovulatory cycle than that during which the E_2 levels were measured, the presence of a competent folliculogenesis reflected by high hormonal response can be traced back to the quality of the embryos/oocytes and hence the presence of a better ovarian reserve *(Table 7.22-7.24 CD)*.

As expected, the number of embryos thawed correlated positively with the number of surviving embryos, but also with the number and grade of the embryos transferred. This association indicated the clinical intention for a better selection opportunity for ET where higher quality embryos might be transferred if they can be selected from a larger cohort. Furthermore, the number of embryos remaining after the index FTER cycle correlated negatively with the grade of the best embryo and, despite not reaching the level of significance, this trend indicated a positive link between the quantitative and qualitative elements of the ovarian response, where higher numbers usually equated with better quality and outcome.

No link was found between the number of embryos transferred and their quality. This was not only because of the Unit policy advising against the transfer of

extra embryos if the quality is less than ideal but also because the majority of embryos transferred were of good grade.

8.1.3 Predictive Role of the Clinical and Embryological Variables of the NC for the Treatment Outcome

When the clinical and embryological parameters of the NC group were evaluated for pregnancy rates, only the number of embryos remaining and mid-luteal P_4 levels were significantly different between outcome groups. Women with a higher number of embryos remaining or exhibiting higher luteal phase P_4 levels tended to have higher pregnancy rates *(Table 7.39-7.42 CD).*

The first association reflects the wider picture of a greater number of embryos being associated with better treatment outcome. Higher embryo availability may also equate with better survival rates and so better embryo quality and treatment outcome. However, having more embryos remaining for future cycles also indicated that successful index treatment was less likely to be preceded by multiple previous replacement cycles.

Although the association is not always acknowledged, higher luteal phase P_4 levels may reflect a more competent corpus luteal function, which is structurally and functionally dependent upon a more competent folliculogenesis. Although the embryos were generated in a previous stimulated cycle, the finding of 'good' follicular development fits a general model of higher ovarian reserve, which should have been even better at a younger female age of the primary fresh IVF treatment cycle. Furthermore, considering that endometrial receptivity is a function of adequate decidualisaton under the effect of P_4, this association finds its biological plausibility both in endometrial and embryological development. Garcia et. al.[550] point out the same contention from a different perspective, stating that inadequate induction of the progesterone receptors as a result of suboptimal folliculogenesis can lead to defective secretory differentiation.

In NC, ET is usually scheduled with reference to the LH surge, determined by serial urinary or serum tests with or without E_2 measurements; confirmation of ovulation by ultrasonography could be added to this protocol. There are no

studies comparing the different cycle monitoring protocols with regard to their efficiency in optimising the timing of ET. In the current study serial serum LH and E_2 measurements were used to detect the onset of LH surge.

It is of note that neither the day of LH surge nor the level of LH and E_2 on the day of LH surge or at its peak differed between the pregnant and non-pregnant cycles, indicating that none of these variables has any prognostic significance on outcome. Therefore, no assumption of outcome and treatment alteration should be based on these follicular phase findings.

With respect to the onset of LH surge, the day of ET had no effect on the probability of pregnancy. Therefore, within the range of 3 to 6 days after the onset of LH surge, no deterioration of the endometrial receptivity was detected. In natural cycles, the endometrium reaches a state of readiness for implantation approximately 7 days after the LH peak, or 5.5 days after ovulation[367]. This maximum endometrial receptivity extends to post-ovulatory day 10, defining the 'window of implantation' between days 19 and 24 of the NC[372]. Pinopode expression linked to the 'implantation window', has been observed at around day 20 of the NC. The entire lifespan of pinopods is a maximum 48 hours, albeit with variation of up to 3 days between individual women[373,374,375]. The current study confirmed that endometrial maturity did not negatively affect the outcome within the range of LH+3 to 6 days. Considering that LH+3-endometrium corresponds to day 1 embryonic growth and LH+6 to day 4, and the transferred embryos are at day 2 of maturity, requiring a further 3 days of maturity in the uterus before implantation, the endometrium would be at LH+6 to 9, within the proposed window of implantation 'LH+6.5 to 11.5'.

The quality of the transferred frozen-thawed embryos was superior in the pregnant women but this did not reach significance in the whole study population; this was also valid when ongoing pregnancy rather than clinical pregnancy was used as the outcome measure. It has been reported that morphologically evaluated embryo quality is the most important clinical factor for successful implantation in FTER[406,483].

It appeared that embryo quality was the key element and this was linked directly to the quality and quantity of the ovarian response in the primary fresh IVF treatment cycle. In this context, the optimum hormonal milieu required for endometrial development and a successful outcome also linked to the ovarian performance with a good margin of flexibility for the 'window of implantation'.

8.1.4 Predictive Role of the Clinical and Embryological Variables of the DRRC for the Treatment Outcome

In the DRRC group, embryo quality was significantly better in the pregnant group but was short of significance in the NC group. Embryo quality evaluated morphologically is shown to be the most important clinical factor for successful implantation of FTER cycles[483]. With ongoing pregnancy as the outcome measure, the difference in embryo quality between the pregnant and non-pregnant groups lost significance but the trend was maintained. This provided further supporting evidence that embryo quality was the critical prognostic variable, and circumstantial evidence that embryo quality assessed anatomically, despite maintaining its deterministic influence over the initial implantation, did not differentiate subsequent miscarriages in FTER cycles *(Table 7.43-7.46CD).*

8.1.5 Comparison of the Clinical and Embryological Variables in the Primary Fresh IVF Treatment Cycles of the NC and DRRC Groups

Clinical and embryological parameters of the primary fresh IVF treatment cycles were compared, to assess whether they differed between couples having NCs or DRRC FTERs *(Table 7.47CD).*

There were no differences between the NC and DRRC groups in terms of female age, duration and aetiology of subfertility, past obstetric history, BMI, smoking and alcohol use, sperm parameters, type of fertilization in the fresh cycle (conventional IVF or ICSI), grade of embryos selected for transfer in the fresh treatment cycle, and distribution of divided and pronuclear stage embryos and the quality of embryos transferred in the FTER cycle.

The duration of the menstrual cycle was 1.5 days longer in the DRRC group than the NC group respectively (28.5 vs 30 days) and although this reached statistical significance it is unlikely to have any clinical relevance in terms of ovulatory potential in either group.

Women in the DRRC group presented with better ovarian reserve and better ovarian response to milder ovarian stimulation in their fresh IVF cycles. Early follicular FSH values and the total gonadotrophin dose used during stimulation was lower in the DRRC group, and E_2 level on the day of hCG injection, number of developing follicles particularly those measuring 10-15mm, total number of follicles aspirated, number of oocytes collected and oocytes used for fertilisation, number of 2-PN embryos created and number of embryos cryopreserved, were all higher in the DRRC group. Because these fresh treatment cycle variables are those that affect FTER cycle outcome, as would be expected the pregnancy rate was higher in the DRRC group (28.6%) than in the NC group (25%) but the difference did not reach statistical significance.

It was of note that in the fresh treatment cycle of the NC group, the number of embryos implanted was higher (p: 0.03) and the ongoing pregnancy rate twice that of the DRRC group (p: 0.2) (Table 7.1, 7.2, 7.5, *Table 7.47CD*). However, it was already noted that the outcome of the fresh treatment cycle did not affect the outcome of subsequent FTER cycles.

The majority of the fresh ET procedures were graded as easy, but women in the NC group had their fresh ET procedures more easily than women in the DRRC group. This may explain why the fresh IVF treatment cycles of the NC group achieved higher pregnancy rates despite having poorer ovarian response. Nevertheless, it was already shown that the grade of difficulty of the fresh ET procedure did not present as a statistically significant confounding factor in analysis of FTER cycles *(Table 7.73-7.75CD).*

Couples randomized or allocated to their treatment cycles on personal preference did not differ in the demographic variables or the clinical and embryological parameters of the primary fresh IVF treatment cycles. Hence,

although the majority of the participating couples were not randomised, all of the relevant prognostic factors found to affect the outcome had equal distribution between the randomised and allocated subjects *(Table 7.61-7.72CD).* Therefore, in statistical terms, an apparent disadvantage to the study design did not introduce a prognostic bias. Despite the domination of good responders in the primary fresh IVF treatment of the DRRC group over the NC group did introduce a potential heterogeneity, the quality of the transferred embryos during FTER in the NC and DRRC groups was similar *(Table 7.77CD).*

In the DRRC group, variations in embryo quality significantly affected the treatment outcome, but not in the NC group. As the quality of the transferred embryos was similar in both the NC and DRRC groups, this may indicate that other aspects of implantation might have assumed a compensatory role in the NC group to offset the variation in embryo quality. Hence, endometrial receptivity might be less compensating in the DRRC group. However, despite this probable vulnerability in the DRRC group their pregnancy rates in FTER were insignificantly higher. This may have been due to the inherent high fertility potential of the embryos in the DRRC group, evidenced by higher ovarian response parameters in the fresh treatment cycle but not detectable by the current anatomical grading of the embryos.

Some authors report higher pregnancy rates in hormonally controlled cycles than in natural cycles, but mostly in small studies[440,445], while others using larger semi-randomized or retrospective studies report a similar outcome in both types of cycles[409,454,478,479,482]. On the contrary, Loh and Leong[485] report a natural cycle pregnancy rate almost twice that of the hormone replacement cycles. With ET on days 14 to 16 of a natural cycle, the pregnancy rate reaches 33%.

The available data from these studies suffers from the methodological problem of comparing two unidentical groups in their response to two different therapeutic regimens. Women with proven ovulation and regular cycles were mostly included in the natural cycles and women with anovulation and irregular cycles in the hormone replacement cycles. Furthermore, variables of the fresh

IVF cycles were rarely taken into consideration limiting the chances of like-to-like comparison. The current study minimised these methodological problems.

8.2 Factors Linked to Embryo Cryo-survival Rate in FTER

In the NC FTER group, the embryo survival rate did not correlate with any of the demographical, clinical, and embryological variables of the primary fresh IVF treatment cycle, or with the serum VEGF and VEGFR levels on the day of ET and number of embryos implanted in FTER cycles *(Table 7.78-7.110CD).* Konc et. al.[551] report that female BMI but not age affects the embryo survival rates. PN stage-embryo survival is shown to be negatively affected by ICSI, possibly caused by disruption of the zona pellucida and vitelline membrane[552]; while no effect on the implanting capacity of the surviving embryos is observed[553].

However, in the DRRC group, embryo survival was higher in cycles where the fresh IVF treatment was characterised by better ovarian response to stimulation. A greater number of developing follicles measuring 16-17mm, and total number of follicles aspirated, oocytes collected, and oocytes used for fertilisation, provided a favourable background for embryos likely to survive the cryopreservation and thawing processes.

This observation gave credit to the contention that better ovarian response to stimulation indicated an inherently better quality embryo, and that this not only affected the treatment outcome of the FTER but also the survival capacity of the embryos during the cryopreservation and thawing processes. As the embryo survival rate was independent of the absolute number of embryos, it was not the high number of embryos that was associated with a higher probability of survival. Furthermore, higher quality embryos were available from the embryo cohorts with higher survival rates and this was valid for both the NC and DRRC groups; yet again the association was more significant in the DRRC group *(Table 7.110CD).* High quality embryos are known to sustain less cryoinjury during cryopreservation, when compared with those of moderate and poor quality[549]. Interestingly, the survival rates themselves did not directly associate with the treatment outcome of the FTER cycles or that of the primary fresh IVF treatment cycle *(Table 7.104-7.109CD).*

Whilst these observations were more significant in the DRRC group, logically they cannot be due to the differences in the treatment protocols as the embryos of both groups were cryopreserved and thawed in accordance with the same protocol, regardless of the way the endometrium was prepared. Furthermore, in both groups the embryo survival rate was the same at 80%.

In the NC and DRRC groups, embryo survival was higher in secondary female subfertility but this approached statistical significance only in the DRRC group. Female smoking and alcohol consumption, stimulation protocol, type of GnRHa and type of fertilisation (IVF or ICSI) did not affect the embryo survival rate.

8.3 Comparison of the Cleavage Stage and Pro-nuclear Stage Embryo

The survival rate was significantly higher in pronuclear stage embryos in the DRRC group and although this trend was also evident in the NC group it did not reach a level of significance. Ongoing pregnancy rates and the number of implanted embryos were significantly higher with pronuclear stage embryos. This effect must partly have been due to the higher survival ability of the embryos cryopreserved at the pronuclear stage. However, a likely explanation was the higher quality of the transferred embryos, which were cryopreserved at pronuclear stage. Nonetheless, this trend failed to reach a level of statistical significance *(Table 7.111-7.119CD).* Salumets et. al.[554] have shown that developmental stage of embryos at freezing has a profound effect on cryo-survival, but has little effect on rates of implantation and ongoing pregnancy rates. The poor survival and elevated miscarriage rates are both high for day 3 when compared with PN embryos.

8.4 Predictive Role of the VEGF, VEGFR, VEGF:VEGFR Levels for the Treatment Outcome

Differences in VEGF and VEGFR concentrations in serum on the day of FTER did not reach a level of statistical significance between pregnant and non-pregnant women and women with ongoing pregnancy and no ongoing pregnancy (Table 7.13). No published literature was available for comparison on this topic.

Despite not reaching a level of statistical significance, higher VEGF and lower VEGFR levels were evident in women who subsequently achieved pregnancy and this distinction became even more evident in those whose pregnancy was ongoing.

Higher VEGF levels indicate a state of activated angiogenetic response and concomitantly lower VEGFR concentrations ensure the maximisation of the biologically active free VEGF molecules for end organ stimulation. Hence, it may be deduced that the treatment cycles with a higher angiogenetic milieu through provision of better tissue oxygenation, were more likely to achieve higher implantation and pregnancy rates. Supporting this observation, a high ratio of VEGF to its receptor was also evident in conception cycles, reflecting the availability of more free VEGF molecules for its biological action (Table 7.15).

Despite not reaching a level of statistical significance, ET cycles destined for ongoing pregnancies had higher VEGF concentrations when compared with those with early miscarriages, reflecting a possible dose response relationship between the intensity of facilitating factors and the successive stages of implantation (Table 7.13). If angiogenetic support to implantation is optimum the process is likely to reach completion and the outcome would be ongoing pregnancy. Alternatively, if the angiogenetic support is suboptimum the implantation process is likely to arrest prematurely and the outcome would be early miscarriage.

8.4.1 VEGF-VEGFR Levels in the NC and DRRC Groups

When compared with NC, VEGF levels were higher and VEGFR levels were lower in DRRC. However, only the latter reached a level of significance. VEGF response also differed between the NC and DRRC groups in relation to the treatment outcome, but at a statistically insignificant level. In the NC group both VEGF and VEGFR levels were lower in the pregnant cycles. In the DRRC group, high VEGF and low VEGFR levels characterised the pregnant cycles. When NCs and DRRCs were compared, VEGF levels were higher in the DRRC if there was pregnancy but lower if there was no pregnancy. These differences

did not reach a level significance (Table 7.16-7.20). When NCs and DRRCs were compared, VEGFR levels were lower in the DRRC. These differences reached a level of significance if there was pregnancy.

In the DRRC group the high VEGF levels might have been induced by the transient local hypoxic state generated by GnRH analogue down-regulation, associated with low oestrogen levels and decreased tissue perfusion. Subsequent exogenous oestrogen supplementation may have further stimulated VEGF levels in the DRRC group, above that achievable by the endogenous oestrogen concentrations of the NC group. Physiological levels of oestrogen are shown to stimulate VEGF production by human epithelial and stromal cell cultures through both oestrogen receptor α and β[555,556]. However, oxygen tension appears to be more important in regulating VEGF synthesis than proposed hormonal effects. Sharkey et. al.[545] show that hypoxic regulation of VEGF expression is a potent feature of endometrial VEGF regulation.

Cycles achieving higher VEGF levels and thus perhaps with better oxygenation potential, appeared to have a higher probability of pregnancy in DRRC. On the contrary, in non-conception cycles of the DRRC group, VEGF levels were particularly low, further reflecting that hypoxia might be the underlying problem. Also in the literature, a high proportion of women (37%) are found to have impaired uterine perfusion in HRT cycles for FTER[228]. In the DRRC group, low VEGF levels appeared to be more detrimental than in the NC group and this was possibly because low VEGF levels indicated a core subgroup of women with a more fundamentally seated hypo-responsiveness in angiogenesis, despite having an exogenous stimulant (E_2) in DRRC. Low VEGF levels in NCs may reflect a state of optimum oxygenation or unsuppressed intact mechanisms may have provided alternative means to support angiogenesis so that pregnancy can still occur with low VEGF levels.

When compared with NC, an insignificantly higher VEGF and significantly lower VEGFR levels in the DRRC, may be the indicative of a more active angiogenetic state. Higher pregnancy rates in the DRRC group have already been pointed out and although this did not reach significance, it supported our

initial contention that better oxygenation potential is linked to better functional capacity and treatment outcome. A possible vulnerability of the DRRC outcome to embryo quality was hypothesised to be due to a probable loss of compensatory mechanisms for low embryo quality at the endometrial level; this may be the inability of DRRC to maintain optimum angiogenetic stimuli and hence, oxygenation in a subgroup of women with low VEGF levels.

8.5 Endometrial Echo-pattern of FTER Cycles

Before the ET procedure, an ultrasonographic assessment of the endometrial echo-pattern and thickness was performed using a transvaginal probe. The endometrial echo-pattern was classified according to the appearance of the hyper- (w) and hypo- (b) echogenic zones of the endometrium relative to the myometrium, and the presence or absence of a distinct midline echo (ml). Three distinct patterns were recognised. The first was dominated by a homogeneously hyperechogenic endometrium with no distinct midline echo 'w'. The second had a homogeneous hyper-echogenic endometrium with a central hypo-echogenic zone but no midline echo 'w-b-w'. The third was characterised by a triple layer of peripheral hyper-echogenic zone at the myometrial junction, separated by inner hypo-echogenic zones and a midline echo 'w-b-ml-b-w'. The central echogenic line 'ml' represents the uterine cavity and the outer hyper echogenic zones 'w' represent the basal layer of the endometrium. Between the two outer zones and the central line, the hypo-echogenic regions 'b' represents the functional layer of the endometrium[384].

The endometrial patterns described as 'w', 'w-b-w' and 'w-b-ml-b-w' did not differ significantly among the different demographic and clinical variables of the female partner and FTER cycles *(Table 7.139, 7.140CD).* These include female age, height, weight, or BMI, duration of subfertility, type and aetiology of subfertility, past obstetric history, duration of menstrual cycle, smoking or alcohol consumption, early follicular phase FSH, day of LH surge, LH and E_2 level on the day of LH surge, ET day, mid-luteal P_4 in NC, E_2 and P_4 level after down-regulation in DRRC, and VEGF and VEGFR concentrations on the day of ET. These observations were valid for the whole study population as well as separately for the NC and DRRC groups. Bustillo et. al.[277] also report no

correlation between the cumulative dose of oestrogen intake or serum oestradiol concentration prior to progesterone administration, and the echogenic pattern of the endometrium.

In the NC FTER group, the 'w-b-w' pattern, which associated with the highest implantation rate per cycle (p: 0.05) and number of previous pregnancies (p: 0.09), also associated with the highest LH level on the day of the surge, the highest maximum-LH level during the surge and the highest mid-luteal P_4 concentrations albeit at a statistically insignificant level.

The favourable echo-pattern 'w-b-w' also associated with the highest serum VEGF and lowest VEGFR level, but although numerical differences were obvious, these did not reach significance. This VEGF-VEGFR pattern may signify the maximum availability of angiogenetic stimuli to the target organs. The pattern 'w-b-ml-b-w', which associated with the lowest number of implanted embryos, also associated with the VEGF-VEGFR pattern possibly indicating the lowest availability of angiogenetic stimuli (low VEGF and high VEGFR). In these associations the significance of the 'w' pattern appeared to fall between the 'w-b-w', and 'w-b-ml-b-w' patterns *(Table 7.140CD).*

Association between high LH and high VEGF concentrations arises from LH stimulation of the VEGF activity[557,558], which is vital in the neovascularisation of the corpus luteum formation and the adequacy of corpus luteum function. In turn, corpus luteum function links to endometrial maturity and function, which requires adequate progesterone and possibly VEGF from the corpus luteum. Nevertheless, human endometrial cells are known to produce VEGF under the stimulatory effect of oestrogen[555,556] and probably also by progesterone, although there are conflicting reports on the latter[545,559,560.561,562].

Therefore, the association between VEGF levels and endometrial growth may be indirect through ovarian function orchestrated by steroid hormones and/or by ovarian angiogenetic stimuli; or direct through local endometrial VEGF production and neovascularisation.

In the DRRC group, these trends were not evident and this is probably due to the loss of natural homeostatic dynamics by down-regulation not being effectively replaced with the hormone replacement. Nevertheless, this apparent deficiency at the endometrial level did not seem to alter the probability of pregnancy in the DRRC group and this, yet again, may be due to a presumed superiority of the embryo cohort in this group.

Endometrial thickness did not differ significantly between different endometrial patterns with mean values of endometrial thickness >9mm in all patterns. As a proxy measure of endometrial growth, endometrial thickness is reported to be unrelated to endometrial pattern on the day of HCG injection[378,389,390,395].

In summary, with the limitation of a significant chance factor in mind, superior progesterone and angiogenetic stimuli appeared to be associated with better endometrial receptivity and this was linked to higher LH stimuli and presumed superior corpus luteal function. This combination was best represented by the 'w-b-w' endometrial echo-pattern and least by the 'w-b-ml-b-w' pattern. Although an association between the endometrial echo-pattern and the number of implanted embryos was demonstrated, no significant differences between conception and non-conception cycles were found. In their ultrasonographic evaluation of the endometrium in women undergoing natural cycle IVF, Ueno et. al.[404] report that endometrial pattern A (homogeneous, hyperechogenic 'w') on the day prior to oocyte collection had a predictive value of 100% for a non-conception cycle but pattern B (mixed, with an outer hyperechogenic and inner hypoechogenic layer 'w-b-w') correlated with a significantly greater proportion of conception than non-conception cycles.

With findings similar to those of the current study, Al Shawaf et. al.[409] report that the methodology of endometrial preparation had no influence on the observed endometrial echo-pattern, because no significant differences were noted between NCs and DRRCs and conception and non-conception cycles. Check et. al.[417] and Alam et. al.[411] also report no significant variations in the endometrial echo-pattern of conception and non-conception cycles after HRT treatment cycles. However, Bustillo et. al.[277], Coulam et. al.[256], and Shapiro et.

al.[412] noted significant differences, with a triple line pattern, the most commonly distinguished in conception cycles, but this is not a consistent observation.

The 'w-b-ml-b-w' pattern indicates a more advanced stage of endometrial development and the negative prognostic effect is likely to be the result of significant asynchrony between embryo and endometrial maturity. If embryos are transferred into a less advanced endometrium the chances of implantation would be higher because advanced embryonic growth allows embryos to implant as soon as the less advanced endometrium gets receptive. Endometrium may quickly become post-mature and closes the implantation window, so less mature embryos may lose the chance of implantation by the time they are ready. Likewise, a lower pregnancy rate is reported if the endometrium pattern is more advanced[397].

8.6 Endometrial Thickness of FTER Cycles

In the NC and DRRC groups, mean endometrial thickness was 9.5 and 9.6 mm respectively *(Table 7.166-7.168CD)*. Although mean values were similar, the distribution around this mean differed. While 7.7% of women in the NC group had an endometrial thickness of 5 mm or less, in the DRRC group this was only 2.5%. Similarly, in the NC group 23% of women had an endometrial thickness of 7 mm or less and in the DRRC group this was only 17%. However, from 7mm upwards towards the thicker endometrium, the distribution in both groups is of a similar pattern. Therefore, for women who would have had a thinner endometrium if in the NC group, exogenous hormone replacement might have corrected this to some extent and a core subgroup of 5% of the study cohort could have benefited from this effect. We argued that there is a threshold stimulatory level of endometrial growth, above which the dose response curve between hormone stimulation and endometrial thickness flattens.

A threshold value for endometrial thickness varying between 5mm and 8mm, below which implantation is exceptional, is common in the literature[391,409]. In the NC group, a larger proportion of women were below this threshold than in the DRRC group, and the expectation would be for lower pregnancy rates in the NC

group. This was, what we observed, but not at a level of significance, as discussed in the previous section.

In the current study, there was only one pregnancy when the endometrium measured <6.6mm (4.8mm) compared with 17 pregnancies when the endometrium measured ≥6.6 mm.

Furthermore, once the minimum endometrial thickness was achieved, there seemed to be no additional benefit with a thicker endometrium. Hence, when the whole spectrum of endometrial values from thinnest to thickest was evaluated, no correlation was observed between endometrial thickness and the average number of embryos implanted in either the NC or DRRC group and mean values of endometrial thickness at the time of ET in pregnant and non-pregnant women, were not statistically different. This was also valid for ongoing pregnancies. Therefore, mean endometrial thickness was not a determinant for the probability of implantation or early miscarriage.

The evidence from the literature is conflicting. While Al Shawaf et. al.[409], Friedler et. al.[410], Coulam et. al.[256] and Bustillo et. al.[277] find no significant differences between conception and non-conception HRT cycles, Check et. al.[417,539], Alam et. al.[411], Abdalla et. al.[391], Schwartz et. al.[406] and Banz et. al.[563], report significant differences, but even these amount to only a 1 to 1.5 mm variation.

Hence, the clinical role of physical assessment of endometrial growth as a surrogate marker for endometrial receptivity must be, at best marginal, when only the extremes of poor growth are seen[288,393,403,405,564]. However, employing a minimal threshold for endometrial thickness is more promising with the assumption that such a crude physical variable only allows a gross 'all or none' assessment, and its sensitivity does not allow fine-tuning of the receptivity evaluation.

8.6.1 Endometrial Thickness and Clinical Variables

Endometrial thickness correlated positively with the E_2 level on the day of LH surge and the stimulatory role of oestrogens on endometrial proliferation and growth is well known[394,540]. A negative correlation observed with the day of LH surge and LH level may be an indirect one, arising from its positive associations with oestrogen. While high oestrogen levels led to a thicker endometrium they may also induce an earlier LH surge. In this contention it is assumed that the relatively early termination of the follicular phase and oestrogen stimulation, before the availability of progesterone with its anti-oestrogenic properties, had less effect on the final endometrial thickness. This assumption was supported by the finding of no correlation between endometrial thickness and the level of luteal phase P_4 in the NC group *(Table 7.145-7.151CD).*

A negative correlation with VEGFR levels reflected the role of angiogenesis in endometrial development. Higher VEGFR levels work to lower the biologically active free VEGF molecules and the subsequent angiogenetic stimuli that seem to lead to thinner endometrium on the day of transfer. A positive correlation was observed between female weight and endometrial thickness and so any future comparative analysis of endometrial thickness should be corrected for female weight. No correlation was observed with endometrial thickness and VEGF levels, indicating that the endometrium may have higher sensitivity to the level of suppression but less to the level of stimulation. Endometrial thickness did not differ with the type or aetiology of subfertility, or with the female smoking or alcohol consumption.

The current study included a series of linear regression analyses to assess the association between endometrial thickness and different clinical variables. As independent predictors, VEGFR and female weight explained only 4% and 3% respectively of the variation in the endometrial thickness and addition of the VEGF levels did not improve the statistical significance of the equation or its predictive power. In the NC group, a further linear regression analysis, using VEGFR, female weight, day of LH surge and maximum LH level as independent variables, revealed the maximum LH level as the only independent predictor.

The final model with maximum LH level had a p value of 0.002 and explained 23% of the variation. This indicated the primacy of the LH stimulation in the successive events of angiogenesis and steroid production leading to endometrial growth.

9 References

1 Federation, C.E.C.O.S., Schwartz, D. and Mayaux, J.M. (1982) Female fecundity as a function of age: results of artificial insemination in 2193 nulliparous women with azoospermic husbands. New Eng. J. Med., **306**, 404-406.

2 Templeton, A., Fraser, C. and Thompson, B. (1990) The epidemiology of infertility in Aberdeen. B.M.J., **301**, 148-152.

3 Gunnell, D.J. and Ewings, P. (1994) Infertility prevalence, needs assessment and purchasing. J Public Health Med., **16**, 29-36.

4 Southam, A.L. (1960) What to do with the "normal" infertile couple. Fertil. Steril., **11**, 543-549.

5 Lenton, E.A., Weston, G.A. and Cooke, I.D. (1977) Long-term follow-up of the apparently normal couple with a complaint of infertility. Fertil. Steril., **28**, 913-919.

6 Collins, J.A., Wrixon, W., Janes, L.B. and Wilson, E.H. (1983) Treatment-independent pregnancy among infertile couples, N. Engl. J. Med., **309**, 1201-1206.

7 Jansen, R.P.S. (1993) Relative infertility: modelling clinical paradoxes Fertil. Steril., **59**, 1041-1045.

8 Templeton, A., Morris, J.K. and Parslow, W. (1996) Factors that affect outcome of in-vitro fertilisation treatment. Lancet, **348**, 1402-1406.

9 Lu, M.C. (1999) Impact of "non-physician factors" on the "physician factor" of in vitro fertilization success: is it the broth, the cooks, or the statistics? Fertil. Steril., **6**, 998-1000.

10 Karande, V.C., Morris, R., Chapman, C., Rinehart, J. and Gleicher, N. (1999) Impact of the "physician factor" on pregnancy rates in a large assisted reproductive technology program: do too many cooks spoil the broth? Fertil. Steril., **71**,1001-1009.

11 Assisted reproductive technology in the United States: 1998 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. (2002) Fertil. Steril., **1**, 18-31.

12 Chapko, K.M., Weaver, M.R., Chapko, M.K., Pasta, D. and Adamson, G.D. (1995) Stability of in vitro fertilization-embryo transfer success rates from the 1989, 1990, and 1991 Clinic-Specific Outcome Assessments. Fertil. Steril., **4,** 757-763.

13 Dobson, R. (2002) Data on IVF clinics show wide variation in success rate. B.M.J., **7362**, 460.

14 Hull, M.G., Eddowes, H.A., Fahy, U., Abuzeid, M.I., Mills, M.S., Cahill, D.J., Fleming, C.F., Wardle, P.G., Ford, W.C. and McDermott, A. (1992) Expectations of assisted conception for infertility. Br. Med. J., **304**,1465-1469.

15 Tan, S.L., Royston, P., Campbell, S., Jacobs, H.S., Betts, J., Mason, B. and Edwards, R.G. (1992) Cumulative conception and livebirth rates after in-vitro fertilisation. Lancet., **8806,** 1390-1394.

16 Roseboom, T.J., Vermeiden, J.P., Schoute, E, Lens, J.W. and Schats, R. (1995) The probability of pregnancy after embryo transfer is affected by the age of the patient, cause of infertility, number of embryos transferred and the average morphology score, as revealed by multiple logistic regression analysis. Hum. Reprod., **11**, 3035-3041.

17 Widra, E.A., Gindoff, P.R., Smotrich, D.B. and Stillman, R.J. (1996) Achieving multiple-order embryo transfer identifies women over 40 years of age with improved in vitro fertilization outcome. Fertil. Steril., **65**,103-108.

18 Minaretzis, D., Harris, D., Alper, M.M., Mortola, J.F., Berger, M.J. and Power, D. (1998) Multivariate analysis of factors predictive of successful live births in in vitro fertilization (IVF) suggests strategies to improve IVF outcome. J. Assist. Reprod. Genet. **6,** 365-371.

19 Commenges-Ducos, M., Tricaud, S., Papaxanthos-Roche, A., Dallay D., Horovitz, J. and Commenges, D. (1998) Modelling of the probability of success of the stages of in-vitro fertilization and embryo transfer: stimulation, fertilization and implantation. Hum. Reprod., **13**, 78-83.

20 Croucher, C.A., Lass, A., Margara, R. and Winston, R.M. (1998) Predictive value of the results of a first in-vitro fertilization cycle on the outcome of subsequent cycles. Hum. Reprod., **13**, 403-408.

21 Padilla, S.L. and Garcia, J.E. (1989) Effect of maternal age and number of in vitro fertilization procedures on pregnancy outcome. Fertil. Steril., **52**, 270–273.

22 Van Kooij, R.J., Looman, C.W., Habbema, J.D., Dorland, M. and Te Velde, E.R. (1996) Age-dependent decrease in embryo implantation rate after in vitro fertilization. Fertil. Steril., **5,** 769-775.

23 Stolwijk, A.M., Zielhuis, G.A., Hamilton, C.J., Straatman, H., Hollanders, J.M., Goverde, H.J., Van Dop, P.A. and Verbeek, A.L. (1996) Prognostic models for the probability of achieving an ongoing pregnancy after in-vitro fertilization and the importance of testing their predictive value. Hum. Reprod.,**10**, 2298-2303.

24 Assisted reproductive technology in the United States and Canada: 1995 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. (1998) Fertil. Steril., **3**, 389-398.

25 Assisted reproductive technology in the United States: 1996 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry.(1999) Fertil. Steril., **5,** 798-807.

26 Assisted reproductive technology in the United States: 1999 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. (2002) Fertil. Steril., **5,** 918-931.

27 De Mouzon, J., Rossin-Amar, B., Bachelot, A., Renon, C. and Devecchi, A. (1998) FIVNAT. Influence of attempt rank in in vitro fertilization. Contracept. Fertil. Steril., **7-8,** 466-472.

28 Abdalla, H.I., Burton, G., Kirkland, A., Johnson, M.R., Leonard, T., Brooks, A.A. and Studd, J.W. (1993) Age, pregnancy and miscarriage: uterine versus ovarian factors. Hum. Reprod., **9**,1512-1517.

29 Yaron, Y., Botchan, A., Amit, A., Kogosowski, A., Yovel, I. and Lessing, J.B. (1993) Endometrial receptivity: the age-related decline in pregnancy rates and the effect of ovarian function. Fertil. Steril., **60**, 314–318.

30 Faddy, M.J. and Gosden, R.G. (1996) A model conforming the decline in follicle numbers to the age of menopause in women. Hum. Reprod., **11**, 1484-1486.

31 Batista, M.C,. Cartledge, T.P., Zellmer, A.W., Merino, M.J., Axiotis, C., Bremner, W.J. and Nieman, L.K. (1995) Effects of aging on menstrual cycle hormones and endometrial maturation. Fertil. Steril., **3,** 492-499.

32 Navot, D., Drews, M.R., Bergh, P.A., Guzman, I., Karstaedt, A. and Scott, R.T. Jr (1994) Age-related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. Fertil. Steril., **61**, 97–101.

33 Battaglia, D.E., Goodwin, P., Klein, N.A. and Soules, M.R. (1996) Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Hum. Reprod., **1**, 2222-2227.

34 Bendiva, C.A., Klingman, I. and Munne, S. (1996) Aneuploidy 16 in human embryos increases significantly with maternal age. Fertil. Steril., **66**, 248-255.

35 Seifer, D.B., Gardiner, A.C., Ferreira, K.A. and Peluso, J.J. (1996) Apoptosis as a function of ovarian reserve in women undergoing in vitro fertilization. Fertil. Steril., **66**, 593-598.

36 Leidy, L.E., Godfrey, L.R. and Sutherland, M.R. (1998) Is follicular atresia biphasic? Fertil. Steril., **70**, 851-859.

37 Henderson, S.A. and Edwards, R.G. (1968) Chiasma frequency and maternal age in mammals. Nature, **218**, 22-28.

38 Polani, P.E. and Crolla, J.A. (1991) A test of the production line hypothesis of mammalian oogenesis. Hum. Genet., **88**, 64-70.

39 Zheng, C.J. and Byers, B. (1992) Oocyte selection: a new model for the maternal-age dependence of Down syndrome. Hum. Genet., **90**, 1-6.

40 Volarcik, K., Sheean, L., Goldfarb, J., Woods, L., Abdul-Karim, F.W. and Hunt, P. (1998) The meiotic competence of in-vitro matured human oocytes is influenced by donor age: evidence that folliculogenesis is compromised in the reproductively aged ovary. Hum. Reprod., **13**, 154-160.

41 Faddy, M.J., Gosden, R.G., Gougeon, A., Richardson, S.J. and Nelson, J.F. (1992) Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum. Reprod., **7**, 1342-1346.

42 Bachelot, A., Pouly, J.L., Devecchi, A., Quenard, A. and de Mouzon, J. (1998) 1997 FIVNAT general balance Contracept. Fertil. Sex., **26**, 463-465.

43 Tan S.L., Doyle, P., Maconochie, N., Edwards, R.G., Balen, A., Bekir, J., Brinsden, P. ande Campbell, S. (1994) Pregnancy and birth rates of live infants after in vitro fertilization in women with an without previous in vitro fertilization pregnancies: a study of eight thousand cycles at one center. Am. J. Obstet. Gynecol., **1**, Pt 1, 34-40.

44 Levy, T., Goldman, J.A., Dicker, D., Ashkenazi, J. and Feldberg, D. (1991) Very early pregnancy wastage in in vitro fertilization and embryo transfer (IVF-ET). J. In Vitro Fert. Embryo Transf., **5,** 250-253.

45 Alsalili, M., Yuzpe, A., Tummon, I., Parker, J., Martin, J., Daniel, S., Rebel, M. and Nisker, J. (1995) Cumulative pregnancy rates and pregnancy outcome after in-vitro fertilization: > 5000 cycles at one centre. Hum. Reprod., **2,** 470-474.

46 Dor, J., Seidman, D.S., Ben-Shlomo, I., Levran, D., Ben-Rafael, Z. and Mashiach, S. (1996) Cumulative pregnancy rate following in-vitro fertilization: the significance of age and infertility aetiology. Hum. Reprod., **2,** 425-428.

47 Guzick, D.S., Wilkes, C. and Jones, H.W. (1986) Cumulative pregnancy rates for in vitro fertilization. Fertil. Steril., **46**, 663-6718.

48 Kovacs, G.T., Rogers, P., Leeton, J.F., Trounson, A.O., Wood, C. and Baker, H.W. (1986) In-vitro fertilization and embryo transfer. Prospects of pregnancy by life-table analysis. Med. J. Aust., **13**, 682-683.

49 Strandell, A., Lindhard, A., Waldenstrom, U., Thorburn, J., Janson, P.O. and Hamberger, L. (1999) Hydrosalpinx and IVF outcome: a prospective, randomized multicentre trial in Scandinavia on salpingectomy prior to IVF. Hum. Reprod., **14**, 2762-2769.

50 Csemiczky, G., Landgren, B.M., Fried, G. and Wramsby, H. (1996) High tubal damage grade is associated with low pregnancy rate in women undergoing in-vitro fertilization treatment. Hum. Reprod. , **11**, 2438-2440.

51 Tummon, I.S., Maclin, V.E., Radwanska, E., Binor, Z. and Dmowski, W. (1988) Occult ovulatory dysfunction in women with minimal endometriosis or unexplained infertility. Fertil. Steril., **50**, 716-720.

52 Dmowski, W., Radwanska, E., Binor, Z. and Rana, N. (1986) Mild endometriosis and ovulatory dysfunction: effect of danazol treatment on success of ovulation induction. Fertil. Steril., **46**, 784-789.

53 Muse, K., Wilson, E.A. and Jawad, M.J. (1986) Prolactin hyperstimulation in response to thyrotropin-releasing hormone in patients with endometriosis. Fertil. Steril., **38**, 419-422.

54 Grant, A. (1966) Additional sterility factors in endometriosis. Fertil. Steril., **72**, 514-519.

55 Croxatto, H.B., Ortiz, M.E., Guiloff, E., Ibarra, A., Salvatierra, A.M., Croxatto, H.D. and Spilman, C.H. (1978) Effect of 15 (S)-15-methyl prostaglandin $F_{2\alpha}$ on human oviductal motility and ovum transport. Fertil. Steril., **30**, 408-414.

56 Soldati, G., Piffaretti-Yanez, A., Campana, A., Marchini, M., Luetri, M. and Balerna, M. (1989) Effect of peritoneal fluid on sperm motility and velocity distribution using objective measurements. Fertil. Steril., **52**, 113-119.

57 Wardle, P.G., Foster, P.A., Mitchell, J.D., McLaughlin, E.A., Sykes, J.A., Corrigan, E., Hull, M.G., Ray, B.D. and McDermott, A. (1986) Endometriosis and IVF: effect of prior therapy. Lancet., **8475**, 276-277.

58 Simon, C., Gutierrez, A., Vidal, A., De los Santos, M.J., Tarin, J.J., Remohi, J. and Pellicer, A. (1994) Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum. Reprod., **4**, 725-729.

59 Matson, P.L. and Yovich, J.L. (1986) The treatment of infertility associated with endometriosis by in vitro fertilization. Fertil. Steril., **46**, 432–434.

60 Arici, A., Oral, E., Bakulmez, O., Duleba, A., Olieve, D.L. and Jones, E.E. (1996) The effect of endometriosis on implantation: results from Yale University in vitro fertilization and embryo transfer program. Fertil. Steril., **65,** 603-607.

61 Diaz, I., Navarro, J., Blasco, L., Simon, C., Pellicer, A. and Remohi, J. (2000) Impact of stage III-IV endometriosis on recipients of sibling oocytes: matched case-control study. Fertil. Steril., **74**, 31-34.

62 Chang, M.Y., Chiang, C.H., Chiu, T.H., Hsieh, T.T. and Soong, Y.K. (1998) The antral follicle count predicts the outcome of pregnancy in a controlled ovarian hyperstimulation/intrauterine insemination program. J. Assist. Reprod. Genet., **15**,12-17.

63 Lenton, E.A., Sexton, L., Lee, S. and Cooke, I.D. (1988) Progressive changes in LH and FSH and LH:FSH ratio in women throughout reproductive life. Maturitas, **10**, 35-43.

64 Navot, D., Rosenwaks, Z. and Margalioth, E.J. (1987) Prognostic assessment of female fecundity. Lancet, **11**, 645-647.

65 Evers, J.L., Slaats, P., Land, J.A., Dumoulin, J.C. and Dunselman, G.A. (1998) Elevated levels of basal estradiol-17beta predict poor response in patients with normal basal levels of follicle-stimulating hormone undergoing in vitro fertilization. Fertil. Steril., **6**, 1010-1014.

66 Cahill, D.J., Prosser, C.J., Wardie, P.G., Ford, W.C.L. and Hull, M.G.R. (1994) Relative influence of serum follicle-stimulating hormone, age and other factors on ovarian response to gonadotrophin stimulation. Br. J. Obstet. Gynaecol., **101**, 999-1002.

67 Toner, J.P., Philput, C.B., Jones, G.S. and Muasher, S.J. (1991) Basal follicle stimulating hormone level is a better predictor of in vitro fertilization performance than age. Fertil. Steril., **55**, 784-791.

68 Scott, R.T., Toner, J.F., Muasher, S.J, Oehninger, S.C., Robinson, S. and Rosenwaks, Z. (1989) Follicle stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. Fertil. Steril., **51**, 651-654.

69 Pearlstone, A.C., Foumet, N., Gambone, J.C., Pang, S.C. and Buyalos, R.P. (1992) Ovulation induction in women age 40 and older: the importance of basal follicle stimulating hormone level and chronological age. Fertil. Steril., **58**, 674-679.

70 El-Toukhy, T., Khalaf, Y., Hart, R., Taylor, A. and Braude, P. (2002) Young age does not protect against the adverse effects of reduced ovarian reserve-an eight year study. Hum. Reprod., **6**, 1519-1524.

71 Oehninger, S., Veeck, L., Lanzendorf, S., Maloney, M., Toner, J. and Muasher, S. (1995) Intracytoplasmic sperm injection: achievement of high pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors. Fertil. Steril., **64**, 977-981.

72 Biljan, M., Buckett, W., Dean, N., Phillips, S.J. and Tan, S.L. (2000) The outcome of IVF-embryo transfer treatment in patients who develop 3 follicles or less. Hum. Reprod., **15**, 2140-2144

73 Roest, J., van Heusden, A.M., Mous, H., Zeilmaker, G.H. and Verhoeff, A. (1996) The ovarian response as a predictor for successful in vitro fertilization treatment after the age of 40 years. Fertil. Steril., **66**, 969-973.

74 Hanoch, J., Lavy, Y., Holzer, H., Hurwitz, A., Simon, A., Revel, A. and Laufer, N. (1998) Young low responders protected from untoward effects of reduced ovarian response. Fertil. Steril., **69**, 1001–1004

75 Hull, M.G.R., Fleming, C.F., Hughes, A.O. and McDermott, A. (1996) The age-related decline in female fecundity: a quantitative controlled study of implanting capacity and survival of individual embryos after in vitro fertilization. Fertil. Steril., **65**, 783-790.

76 Grimbizis, G., Vandervorst, M., Camus, M., Tournaye, H., Van Steirteghem, A. and Devroey, P. (1998) Intracytoplasmic sperm injection: results in women older than 39, according to age and the number of embryos replaced in selective or non-selective transfers. Hum. Reprod., **13**, 884-889.

77 Bancsi, L.F., Huijs, A.M., den Ouden, C.T., Broekmans, F.J., Looman, C.W., Blankenstein, M.A. and te Velde, E.R. (2000) Basal follicle-stimulating hormone levels are of limited value in predicting ongoing pregnancy rates after in vitro fertilization. Fertil. Steril., **3,** 552-557.

78 Mikkelsen, A.L., Andersson, A.M., Skakkebaek, N.E. and Lindenberg, S. (2001) Basal concentrations of oestradiol may predict the outcome of in-vitro maturation in regularly menstruating women. Hum. Reprod., **16**, 862–867.

79 Akande, V.A., Fleming, C.F., Hunt, L.P., Keay, S.D. and Jenkins, J.M. (2002) Biological versus chronological ageing of oocytes, distinguishable by raised FSH levels in relation to the success of IVF treatment. Hum. Reprod., **8**, 2003-2008.

80 Zenzes, M.T., Wang, P. and Casper, R.F. (1992) Chromosome status of untransferred (spare) embryos and probability of pregnancy after in-vitro fertilisation. Lancet, **340**, 391-394.

81 Roberts, C.G. and O'Neill, C. (1995) Increase in the rate of diploidy with maternal age in unfertilized in-vitro fertilization oocytes. Hum. Reprod., **10**, 2139–2141.

82 Benadiva, C.A., Kligman, I. and Munné, S. (1996) Aneuploidy 16 in human embryos increases significantly with maternal age. Fertil. Steril., **66**, 248–255.

83 Brown, J.R., Liu, H-C, and Sewitch, K.F. (1997) Variability of day 3 follicle-stimulating hormone levels in eumenorrhoeic women. J. Reprod. Med., **40**, 620-624.

84 Ebrahim, A., Rienhardt, G. and Morris, S. (1993) Follicle stimulating hormone levels on cycle day 3 predict ovulation stimulation response. J. Assist. Reprod. Genet., **10,** 130-136.

85 Scott, R.T., Hofmann, G.E., Oehninger, S. and Muasher, S.J. (1990) Intercycle variability of day 3 follicle-stimulating hormone levels and its effect on stimulation quality in in vitro fertilization. Fertil. Steril., **53,** 297-302.

86 Martin, J.S., Nisker, J., Tummon, I., Daniel, S., Auckland, J. and Feyles, V. (1996) Future in vitro fertilization pregnancy potential of women with variably elevated day 3 follicle-stimulating hormone levels. Fertil. Steril., **65**, 1238-1240.

87 Barnhart, K. and Osheroff, J. (1998) Follicle stimulating hormone as a predictor of fertility. Curr Opin Obstet Gynecol., **3**, 227-232.

88 Sherman, B.M. and Korenman, S.G. (1975) Hormonal characteristics of the human menstrual cycle throughout reproductive life. J. Clin. Invest., **55**, 699-706.

89 Sherman, B.M, West, J.H. and Korenman, S.G. (1976) The menopausal transition: analysis of LH, FSH, esiradiol, and progesterone concentrations during menstrual cycles of older women. J. Clin. Endocrinol. Metab., **42**, 619-636.

90 Templeton, A. and Morris, J.K. (1998) Reducing the risk of multiple births by transfer of two embryos after in vitro fertilisation. N. Engl. J. Med., **339,** 573-575.

91 Abdalla, H.I., Gearon, C. and Wren, M. (2000) Swedish in-vitro fertilisation study. Lancet, 355, 844-845.

92 Staessen, C., Camus, M., Bollen, N., Devroey, P. and Van Steirteghem, A. (1992) The relationship between embryo quality and the occurrence of multiple pregnancies. Fertil. Steril., **57,** 626–630.

93 Staessen, C., Nagy, Z.P., Liu, J., Janssenswillen, C., Camus, M., Devroey, P. and Steirteghem, A.C. (1995) One year's experience with elective transfer of two good quality embryos in the human in-vitro fertilization and intracytoplasmic sperm injection programmes. Hum. Reprod., **12,** 3305-3312.

94 Schieve, L.A., Peterson, H.B., Meikle, S.F., Jeng, G., Danel, I., Burnett, N.M. and Wilcox, L.S. (1999) Live-birth rates and multiple-birth risk using in vitro fertilization. J.A.M.A., **19**, 1832-1838.

95 Biggers, J.D. (1988) Fecundability. Ann. N. Y. Acad. Sci., **541**,706-714.

96 Hu, Y., Maxson, W.S., Hoffman, D.I., Ory, S.J., Eager, S., Dupre, J. and Lu, C. (1998) Maximizing pregnancy rates and limiting higher-order multiple conceptions by determining the optimal number of embryos to transfer based on quality. Fertil. Steril. , **4**, 650-657.

97 Elsner, C.W., Tucker, M.J., Sweitzer, C.L., Brockman, W.D., Morton, P.C., Wright, G. and Toledo, A.A. (1997) Multiple pregnancy rate and embryo number transferred during in vitro fertilization. Am. J. Obstet. Gynecol., **2,** 350-355.

98 Senoz, S., Ben-Chetrit, A. and Casper, R.F. (1997) An IVF fallacy: multiple pregnancy risk is lower for older women. J. Assist. Reprod. Genetics, **14**,192-198.

99 Staessen, C., Janssenswillen, C., Van den Abbeel, E., Devroey, P. and Van Steirteghem, A.C. (1993) Avoidance of triplet pregnancies by elective transfer of two good quality embryos. Hum. Reprod., **10**,1650–1653.

100 Licciardi, F., Berkeley, A.S., Krey, L., Grifo, J. and Noyes, N. (2001) A two- versus three-embryo transfer: the oocyte donation model. Fertil. Steril., **3**, 510-513.

101 Ozturk, O. and Templeton, A. (2002) In-vitro fertilisation and risk of multiple pregnancy. Lancet., **9302**, 232.

102 Murdoch, A.P. (1998) How many embryos should be transferred? Hum. Reprod., **10**, 2666-2670.

103 Templeton, A. (2000) Avoiding multiple pregnancies in ART. Replace as many embryos as you like—one at a time. Hum. Reprod., **15,**1662-1665.

104 Erenus, M., Zouves, C., Rajamahendran, P., Leung, S., Fluker, M. and Gomel, V. (1991) The effect of embryo quality on subsequent pregnancy rates after in vitro fertilization. Fertil. Steril., **56**, 707-710.

105 Van Royen, E., Mangelschots, K. and De Neubourg, D. (1999) Characterisation of a top quality embryo, a step towards single-embryo transfer. Hum. Reprod., **14,** 2345-2349.

106 Van Royen, E., Mangelschots, K., Neubourg, D.D, Laureys, Ryckaert, G. and Gerris, J. (2001) Calculating the implantation potential of day 3 embryos in women younger than 38 years of age: a new model. Hum. Reprod., **2**, 326-332.

107 Van Blerkom, J., Antczak, M. and Schrader, R. (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content offollicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum. Reprod., **12**, 1047-1055.

108 Ng, S.T., Chang, T.H. and Wu, J.T.C. (1999) Prediction of the rates of fertilization, cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program. Fertil. Steril., **72**, 412-417.

109 Ebner, T., Moser, M., Yaman, C., Feichtinger, O., Hartl, J. and Tews, G. (1999) Elective transfer of embryos selected on the basis of first polar body morphology is associated with increased rates of implantation and pregnancy. Fertil. Steril., **4,** 599-603.

110 Ebner, T., Yaman, C., Moser, M., Sommergruber, M., Feichtinger, O. and Tews, G. (2000) Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. Hum. Reprod., **2,** 427-430.

111 Fulka, J., Karnikova, L. and Moor, R.M. (1998) Oocyte polarity: ICSI, cloning and related techniques. Hum. Reprod., **13**, 3303–3305

112 Scott, L. and Smith, S. (1998) The successful use of pronuclear embryo transfers the day following oocyte retrieval. Hum. Reprod., **13**, 1003-1013.

113 Tesarik, J. and Greco, E. (1999) The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum. Reprod., **14**, 1318-1323

114 Cummins, J.M., Breen, T.M. and Harrison, K.L. (1986) A formula for scoring human embryo growth rates in in vitro fertilisation: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. J. In Vitro Fert. Embryo Transf., **3**, 284-295.

115 Steer, C.V., Campbell, S., Tan, S.L., Crayford, T., Mills, C., Mason, B.A. and Collins, W.P. (1992) The use of transvaginal color flow imaging after in vitro fertilization to identify optimum uterine conditions before embryo transfer. Fertil. Steril., **57,** 372-376.

116 Giorgetti, C., Terriou, P., Auquier, P., Hans, E., Spach, J.L., Salzmann, J. and Roulier, (1995) R. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. Hum. Reprod., **9**, 2427-2431.

117 Desai, N.N., Goldstein, J., Rowland, D.Y. and Goldfarb, J.M. (2000) Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. Hum. Reprod., **10**, 2190-2196.

118 Gerris, J., De Neubourg, D., Mangelschots, K., Van Royen, E., Van de Meerssche, M. and Valkenburg, M. (1999) Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. Hum. Reprod., **10**, 2581-2587.

119 Kahraman, S., Yakin, K., Donmez, E., Samli, H., Bahce, M., Cengiz, G., Sertyel, S., Samli, M. and Imirzalioglu, N. (2000) Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection. Hum. Reprod., **11**, 2390-2393.

120 Shoukir, Y., Campana, A. and Farley, T (1997) Early cleavage of in vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum. Reprod., **12**, 1531-1536.

121 Devreker, F., Pogonici, E., De Maertelaer, V., Revelard, P., Van den Bergh, M. and Englert, Y. (1999) Selection of good embryos for transfer depends on embryo cohort size: implications for the 'mild ovarian stimulation' debate. Hum. Reprod., **12**, 3002-3008.

122 Hsu, M.I., Mayer, J., Aronshon, M., Lanzendorf, S., Muasher, S., Kolm, P. and Oehninger, S. (1999) Embryo implantation in in vitro fertilization and intracytoplasmic sperm injection: impact of cleavage status, morphology grade, and number of embryos transferred. Fertil. Steril., **4**, 679-685.

123 McKiernan, S.H. and Bavister, B.D. (1994) Timing of development is a critical parameter for predicting successful embryogenesis. Hum. Reprod., **12**, 2123-2129.

124 Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P.G. and Campana, A (1998). Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum. Reprod., **1**, 182-187.

125 Braude, P., Bolton, V. and Moore, S. (1998) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature, **332**, 459-461.

126 Ziebe, S., Petersen, K., Lindenberg, S., Andersen, A.G., Gabrielsen, A. and Andersen, A.N. (1997) Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum. Reprod., **7,** 1545-1549.

127 Buster, J.E., Bustillo, M., Rodi, I.A., Cohen, S.W., Hamilton, M., Simon, J.A., Thorneycroft, I.H. and Marshall, J.R. (1985) Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. Am. J. Obstet. Gynecol., **2**, 211-217.

128 Jurisicova, A., Varmuza, S. and Casper, R.F. (1996) Programmed cell death and human embryo fragmentation. Molecular Hum. Reprod., **2**, 93-98.

129 Warner, C.M., Cao, W., Exley, G.E, McElhinny, A.S, Alikani, M., Cohen, J., Scott, R.T. and Brenner, C.A. (1998) Genetic regulation of egg and embryo survival. Hum. Reprod., **3**, 178-190.

130 Alikani, M., Cohen, J., Tomkin, G., Garrisi, G.J., Mack, C. and Scott, R.T. (1999) Human embryo fragmentation in vitro and its implications for pregnancy and implantation. Fertil. Steril., **5**, 836-842.

131 Ebner, T., Yaman, C., Moser, M., Sommergruber, M., Pölz, W. and Tews, G. (2001) Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome Fertil. Steril., **76,** 2, 281-285.

132 Winston, N.J., Braude, P.R., Pickering, S.J., George, M.A., Cant, A., Currie, J. and Johnson, M.H. (1991) The incidence of abnormal morphology and nucleocytoplasmic ratios in 2-, 3- and 5-day human pre-embryos. Hum. Reprod., **1**, 17-24.

133 Hardy, K., Winston, R.M.L and Handyside, A.H. (1993) Binucleate blastomeres in preimplantation human embryos in vitro: failure of cytokinesis during early cleavage. J. Reprod. Fertil., **98,** 549–558.

134 Pickering, S.J., Taylor, A., Johnson, M.H. and Braude, P.R. (1995) An analysis of multinucleated blastomere formation in human embryos. Hum. Reprod., **7,**1912-1922.

135 Jackson, K.V., Ginsburg, E.S., Hornstein, M.D., Rein, M.S. and Clarke, R.N. (1998) Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in vitro fertilization-embryo transfer cycles. Fertil. Steril., **1**, 60-66.

136 Angell, R.R., Sumner, A.T., West, J.D., Thatcher, S.S., Glasier, A.F. and Baird, D.T. (1987) Post-fertilization polyploidy in human preimplantation embryos fertilized in-vitro. Hum. Reprod., **8**, 721-727.

137 Tesarik, J., Kopecny, V., Plachot, M. and Mandelbaum, J. (1987) Ultrastructural and autoradiographic observations on multinucleated blastomeres of human cleaving embryos obtained by in-vitro fertilization. Hum. Reprod., **2**, 127-136.

138 Munne, S. and Cohen, J. (1993) Unsuitability of multinucleated human blastomeres for preimplantation genetic diagnosis. Hum. Reprod., **8,** 1120-1125.

139 Pelinck, M.J., De Vos, M., Dekens, M., Van der Elst, J., De Sutter, P. and Dhont, M. (1998) Embryos cultured in vitro with multinucleated blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic sperm injection. Hum. Reprod., **4**, 960-963.

140 Roth, R.J., Stanford, N. and Majerus, P.W. (1975) Acetylation of prostaglandin synthetase by aspirin. Proc. Natl. Acad. Sci., **72**, 3073-3067.

141 DeWitt, D.L., el-Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armstrong, R.L. and Smith, W.L. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. J. Biol. Chem., **265**, 5192-5198.

142 Serhan, C.N. (1997) Lipoxins and novel aspirin-triggered 15-epi-lipoxins [ATL]: a jungle of cell–cell interactions or a therapeutic opportunity?. Prostaglandins, **53**, 107-137.

143 Chiang, C.H., Hsieh, T.T., Chang, M.Y., Shiau, C.S., Hou, H.C., Hsu, J.J., Soong, Y.K..(2000) Prediction of pregnancy rate of in vitro fertilization and embryo transfer in women aged 40 and over with basal uterine artery pulsatility index. J. Assist. Reprod. Genet.,**8**, 409-414.

144 Meade, E.A., Smith, W.L., and DeWitt, D.L. (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem., **268**, 6610-6614.

145 Catella-Lawson, F. and Crofford, L.J. (2001) Cyclooxygenase inhibition and thrombogenicity. Am. J. Med., **110**, 28S-32S.

146 Patrono, C. (1994) Aspirin as an antiplatelet drug. N. Engl. J. Med., **330**, 1287-1294.

147 Loew, D. and Vinazzer, H. (1976) Dose-dependent influence of acetylsalicylic acid on platelet functions and plasma coagulation factors. Haemostasis, **5**, 239–249.

148 Bjornsson, T.D., Schneider, D.E. and Berger, H. (1989) Aspirin acetylates fibrinogen and enhances fibrinolysis. Fibrinolytic effect is independent of changes in plasminogen activator levels. J. Pharmacol. Exp. Ther., **250**, 154-161.

149 Fatah, K., Beving, H., Albåge, A., Ivert, T. and Blombäck, M. (1996) Acetylsalicylic acid may protect the patient by increasing fibrin gel porosity. Is withdrawing of treatment harmful to the patient? Eur. Heart J., **17**, 1362–1366.

150 Mahmood, M., Zuberi, H.S. and Ashfaq, M.K. (2001) Nitric oxide mediated effect of cyclo-oxygenase inhibitors. J. Pak. Med. Assoc., **51**, 28-31.

151 Cronstein, B.N., Van de Stouwe, M., Druska, L., Levin, R.I., Weissmann, G. (1994) Nonsteroidal antiinflammatory agents inhibit stimulated neutrophil adhesion to endothelium: adenosine dependent and independent mechanisms. Inflammation, **18**, 323-335.

152 Van Jaarsveld, H., Kuyl, J.M., van Zyl, G.F. and Barnard, H.C. (1994) Salicylate in the perfusate during ischemia/reperfusion prevented mitochondrial injury. Res. Commun. Mol. Pathol. Pharmacol., **86**, 287-295.

153 Colantoni, A., de Maria, N., Caraceni, P., Bernardi, M., Floyd, R.A. and Van Thiel, D.H. (1998) Prevention of reoxygenation injury by sodium salicylate in isolated-perfused rat liver. Free Radic. Biol. Med., **25**, 87-94.

154 Abramson, S.B. and Weissmann, G. (1989) The mechanisms of action of nonsteroidal antiinflammatory drugs. Arthritis Rheum., **32**, 1-9.

155 Tegeder, I., Pfeilschifter, J. and Geisslinger, G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J., **15**, 2057-2072.

156 Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. and DuBois, R.N. (1998) Cyclooxygenase Regulates Angiogenesis Induced by Colon Cancer Cells. Cell., **93**, 705-716

157 Vainio, M., Mäenpää, J., Riutta, A., Ylitalo, P., Ala-Fossi, S.L. and Tuimala, R. (1999) In the dose range of 0.5–2.0 mg/kg, acetylsalicylic acid does not affect prostacyclin production in hypertensive pregnancies. Acta. Obstet. Gynecol. Scand., **78**, 82-88.

158 Patrignani, P., Filabozzi, P. and Patrono, C. (1982) Selective cumulative inhibition of platelet thromboxane production by low-dose aspririn in healthy subjects. J. Clin. Invest., **69**, 1366-1372.

159 Fitzgerald, G.A., Oates, J.A., Hawiger, J., Maas, R.L., Roberts, L.J., Lawson, J.A. and Brash, A.R. (1983) Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. J. Clin. Invest., **71**, 676–688.

160 Lorenz, R.L., Boehlig, B., Uedelhoven, W.M. and Weber, P.C. (1989) Superior antiplatelet action of alternate day pulsed dosing versus split dose administration of aspirin. Am. J. Cardiol., **64**, 1185-1188.

161 Feldman, M., Cryer, B., Rushin, K. and Betancourt, J. (2001) A comparison of every-third-day versus daily low-dose aspirin therapy on serum thromboxane concentrations in healthy men and women. Clin. Appl. Thromb. Hemost., **7**, 53-57.

162 Tulppala, M., Marttunen, M. and Soderstrom-Anttila, V. (1997) Low dose aspirin in prevention of miscarriage in women with unexplained or autoimmune related recurrent miscarriage: effect on prostacyclin and thromboxane A2 production. Hum. Reprod., **12**, 1567-1572.

163 Briggs, S. (1998) Drugs in Pregnancy and Lactation: a reference guide to fetal and neonatal risk. Baltimore. Wiliams and Wilkins, 5th edition, 73-81.

164 Slone, D., Siskind, V., Heinonen, O.P., Monson, R.R., Kaufman, D.W. and Shapiro, S. (1976) Aspirin and congenital malformations. Lancet, **7974**, 1373-1375.

165 Kozer, E., Nikfar, S., Costei, A., Boskovic, R., Nulman, I. and Koren, G. (2002) Aspirin consumption during the first trimester of pregnancy and congenital anomalies: a meta-analysis. Am. J. Obstet. Gynecol., **6**, 1623-1630.

166 Werler, M.M., Sheehan, J.E. and Mitchell, A.A. (2002) Maternal medication use and risks of gastroschisis and small intestinal atresia. Am. J. of Epidemiol., **155**, 26-31.

167 Nielsen, G.L., Sorensen, H.T., Larsen, H. and Pedersen, L. (2001) Risk of adverse birth outcome and miscarriage in pregnant users of non-steroidal anti-inflammatory drugs: population based observational study and case-control study. Br. Med. J., **7281**, 266-270.

168 Li, D.K., Liu, L. and Odouli, R. (2003) Exposure to non-steroidal anti-inflammatory drugs during pregnancy and risk of miscarriage: population based cohort study. Br. Med. J., **327**, 368.

169 Lind, S.E. (1991) The bleeding time does not predict surgical bleeding. Blood, **77**, 2547-2552.

170 Reasbeck, P.G., Rice, M.L. and Reasbeck, J.C. (1989) Double-blind controlled trial of indomethacin as an adjunct to narcotic analgesia after major abdominal surgery. Lancet, **33**, 498–501.

171 Wierod, F.S., Frandsen, N.J., Jacobsen, J.D. Hartvigsen, A. and Olsen, P.R. (1998) Risk of haemorrhage from transurethral prostatectomy in acetylsalicylic acid and NSAID-treated patients. Scand. J. Urol. Nephrol., **32**, 120-122.

172 Lindgren, U. and Djupsjo, H. (1985) Diclofenac for pain after hip surgery. Acta. Anaesthesiol. Scand., **56**, 28–31.

173 Bricker, S.R., Savage, M.E. and Hanning, C.D. (1987) Peri-operative blood loss and non-steroidal anti-inflammatory drugs: an investigation using diclofenac in patients undergoing transurethral resection of the prostate. Eur. J. Anaesthesiol., **4**, 429-434.

174 Rorarius, M.G., Baer, G.A., Metsa-Ketala, T., Miralles, J., Palomaki, E. and Vapaatalo, H. (1989) Effects of peri-operatively administered diclofenac and indomethacin on blood loss, bleeding time and plasma prostanoids in man. Eur. J. Anaesthesiol., **6**, 335-342.

175 Belisle S. and Hardy, J.F. (1996) Hemorrhage and the use of blood products after adult cardiac operations: myths and realities. Ann. Thorac. Surg., **62**, 1908-1917.

176 Derry, S. and Loke, Y.K. (2000) Risk of gastrointestinal haemorrhage with long term use of aspirin: meta analysis. Br. Med. J., **321**, 1183-1187.

177 Sibai, B.M., Caritis, S.N., Thom, E., Shaw, K. and McNellis, D. (1995) Low-dose aspirin in nulliparous women: safety of continuous epidural block and correlation between bleeding time and maternal-neonatal bleeding complications. National Institute of Child Health and Human Developmental Maternal-Fetal Medicine Network. Am. J. Obstet. Gynecol., **5** ,1553-1557.

178 Di Simone, N., Ferrazzani, S., Castellani, R., De Carolis, S., Mancuso, S. and Caruso, A. (1997) Heparin and low-dose aspirin restore placental human chorionic gonadotrophin secretion abolished by antiphospholipid antibody-containing sera. Hum. Reprod., **12**, 2061-2065.

179 Di Simone, N., Caliandro, D., Castellani, R., Ferrazzani, S., De Carolis S. and Caruso, A. (1999) Low-molecular weight heparin restores in vitro trophoblast invasiveness and differentiation in presence of immunoglobulin G fractions obtained from patients with antiphospholipid syndrome. Hum. Reprod., **14**, 489–495.

180 Pierro, E., Cirino, G., Bucci, M.R., Lazzarin, N., Andreani, C.L., Mancuso, S., Lanzone, A. and Navarra, P. (1999) Antiphospholipid antibodies inhibit prostaglandin release by decidual cells of early pregnancy: possible involvement of extracellular secretory phospholipase A2. Fertil. Steril., **71**, 342-346.

181 Adler R.R., Ng A.K. and Rote N.S. (1995) Monoclonal antiphosphatidylserine antibody inhibits intercellular fusion of the choriocarcinoma line. Biol. Reprod., **53**, 905-910.

182 Greaves, M. (1999) Antiphospholipid antibodies and thrombosis. Lancet, **353**, 1348-1353.

183 Abramowsky, C., Vegas, M., Swinenart, G. and Gyves, M. (1980) Decidual vasculopathy in the placenta in lupus erythematosus. Engl. N. Med. J., **303**, 668-672.

184 De Wolf, F., Carreras, L., Moerman, P., Vermylen J., Van Assche, A., and Reanaer, M. (1982) Decidual vasculopathy and extensive placental infarction in a patient with repeated thromboembolic accidents, recurrent fetal loss, and a lupus anticoagulant. Am. J. Obstet. Gynecol., **142**, 829-834.

185 Out, H.J., Bruinse, H.W., Christiaens, G.C., van Vliet, M., Meilof, J.F., de Groot, P.G., Smeenk, R.J. and Derksen, R.H. (1991) Prevalence of antiphospholipid antibodies in patients with fetal loss. Ann. Rheum. Dis., **50**, 553-557.

186 Schorer, A., Wickham, N. and Watson, K. (1989) Lupus anticoagulant induces a selective defect in thrombin-mediated endothelial prostacyclin release and platelet aggregation. Br. J. Haematol., **71**, 399-407.

187 Meroni, P., Papa, N., Beltrami, B., Tincani, A., Balestrieri, G. and Krilis, S. (1996) Modulation of endothelial cell function by antiphospholipid antibodies. Lupus, **5**, 448-450.

188 Peaceman, A.M. and Rehnberg, K. (1995) The effect of aspirin and indomethacin on prostacyclin and thromboxane production by placental tissue incubated with immunoglobulin G fractions from patients with lupus anticoagulant. Am. J. Obstet. Gynecol., **173**, 1391-1396.

189 Zangari, M., Lockwood, C., Scher, J. and Rand, J. (1997) Prothrombin activation fragment (F1.2) is increased in pregnant patients with antiphospholipid antibodies. Thromb. Res., **85**, 177-183.

190 Rote, N.S. (1996) Antiphospholipid antibodies and recurrent pregnancy loss. Am. J. Reprod. Immunol., **35**, 402–407.

191 Di Simone, N. (1995) In vitro effect of antiphospholipid antibody containing sera on basal and gonadotrophin-releasing hormone-dependant human chorionic gonadotrophin release by cultured trophoblast cells. Placenta, **16**, 75–83.

192 Shurtz-Swirski, R., Inbar, O., Blank, M., Cohen, J., Bakimer, R. and Shoenfeld, Y. (1993) In vitro effect of anticardiolipin autoantibodies upon total and pulsatile placental hCG secretion during early pregnancy. Clin. Exp. Rheumatol., **11**, 373-379.

193 Fishman, P., Falach-Vaknin, E., Sredni, B., Meroni, P.L., Tincani, A., Dicker, D., Shoenfeld, Y. (1996) Aspirin-interleukin-3 interrelationships in patients with anti-phospholipid syndrome. Am. J. Reprod. Immunol., **35**,80-84.

194 Bevers, E. (1991) Lupus anticoagulant IgG's (LA) are not directed to phopholipids only but to a complex of lipid-bound human prothrombin. Thromb. Haemostas., **66**, 629-632.

195 Rai, R., Regan, L., Clifford, D.K., Pickering, W., Dave, M. and Mackie, I. (1995) Antiphospholipid antibodies and beta(2)-glycoprotein-I in 500 women with recurrent miscarriage:results of a comprehensive screening approach. Hum. Reprod.,**10**, 2001-2005.

196 Rai, R., Cohen, H. and Dave, M. (1997) Randomized controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies) B.M.J., **314**,253-257.

197 Kutteh, W.H. (1996) Antiphospholipid antibodyassociated recurrent prengancy loss: Treatment with heparin and low-dose aspirin is superior to low-dose aspirin alone. Am. J. Obstet. Gynecol., **174**, 1584-1589

198 Fishman, P., Falach-Vaknin, E., Sredni, B.M.P., Rudniki, C. and Shoenfeld, Y. (1995) Aspirin modulates interleukin-3 production: additional explanation for the preventive effects of aspirin in antiphospholipid syndrome. J. Rheumatol., **22**, 1086–1090.

199 Ermel, L.D., Marshburn, P.B. and Kutteh, W.H. (1995) Interaction of heparin with antiphospholipid antibodies (APA) from the sera of women with recurrent pregnancy loss (RPL). Am. J. Reprod. Immunol., **33**, 14-20.

200 Shushan, A. and Schenker, J.G. (1992) Immunological factors in infertility. American Journal of Reproductive Immunology, **28**, 286-287.

201 Gleicher, N. and El-Roeiy, A. (1988) The reproductive autoimmune failure syndrome. Am. J. Obstet. Gynecol., **159**, 223–227.

202 Sher, G., Feinman, M., Zouves, C., Kuttner, G., Maassarani, G., Salem, R., Matzner, W., Ching, W. and Chong, P. (1994) High fecundity rates following in-vitro fertilization and embryo transfer in antiphospholipid antibody seropositive women treated with heparin and aspirin. Hum. Reprod., **9**, 2278-2283.

203 Gleicher, N., El-Roeiy, A., Confino, E. and Friberg, J. (1989) Reproductive failure because of autoantibodies: unexplained infertility and pregnancy wastage Am. J. Obstet. Gynecol., **160**, 1376-1385.

204 Taylor, P.V., Campbell, J.M. and Scott, J.S. (1989) Presence of autoantibodies in women with unexplained infertility Am. J. Obstet. Gynecol., **161**, 377-379.

205 Birkenfeld, A., Mukaida. T., Minichiello, L., Jackson, M., Kase, N.G. and Yemini, M. (1994) Incidence of autoimmune antibodies in failed embryo transfer cycles. Am. J. Reprod. Immunol., **31**, 65-68.

206 Geva, E., Yaron, Y., Lessing, J.B., Yovel, I., Vardinon, N., Buike, M. and Amit, A. (1994) Circulating autoimmune antibodies may be responsible for implantation failure in m vitro fertilization Fertil. Steril., **62**, 802-806.

207 Birdsall, M.A., Lockwood, G.M., Ledger, W.L., Johnson, P.M. and Chamley, L.W. (1996) Antiphospholipid antibodies in women having in-vitro fertilization. Hum. Reprod., **11**, 1185-1189.

208 Kaider, B.D., Price, D.E., Roussev, R.G. and Coulam, C.B. (1996) Antiphospholipid antibody prevalence in patients with IVF failure. Am. J. Reprod. Immunol., **35**, 388-393.

209 Denis, A.L., Guido, M., Adler, R.D., Bergh, P.A., Brenner, C. and Scott, R.T. Jr. (1997) Antiphospholipid antibodies and pregnancy rates and outcome in in vitro fertilization patients. Fertil. Steril., **67**, 1084-1090.

210 Kutteh, W.H., Yetman, D.L., Chantilis, S.J. and Crain, J (1997) Effect of antiphospholipid antibodies in women undergoing in-vitro fertilization: role of heparin and aspirin. Hum. Reprod., **12**, 1171-1175.

211 Stern, C., Chamley, L., Hale, L., Kloss, M., Speirs, A. and Baker, H. (1998) Antibodies to β2 glycoprotein I are associated with in vitro fertilization implantation failure as well as recurrent miscarriage: results of a prevalence study. Fertil. Steril., **70**, 938–944

212 Eldar-Geva, T., Wood, C., Lolatgis, N., Rombauts, L., Kovacs, G. and Fuscaldo, J., Trounson, A.O. (1999) Cumulative pregnancy and live birth rates in women with antiphospholipid antibodies undergoing assisted reproduction. Hum. Reprod., **14**, 1461-1466.

213 Egbase, P.E., Al Sharhan, M., Diejomaoh M. and Grudzinskas, J.G. (1999) Antiphospholipid antibodies in infertile couples with two consecutive miscarriages after in-vitro fertilization and embryo transfer Hum. Reprod., **14**, 1483-1148.

214 Matsubayashi, H., Sugi, T., Arai, T., Kondo, A., Suzuki, T., Izumi, S., McIntyre, J.A. and Makino, T. (2001) Different antiphospholipid antibody specificities are found in association with early repeated pregnancy loss versus recurrent IVF-failure patients. Am. J. Reprod. Immunol., **46**, 323-329.

215 Fisch, B., Fried, S., Manor, Y., Ovadia, J., Witz, I.P. and Yron, I. (1995) Increased Antiphospholipid antibody activity in in vitro fertilization patients is not treatment-dependent but rather an inherent characteristic of the infertile state. Am. J. Reprod. Immunol., **34**, 370-374.

216 Chilcott, I.T., Margara, R.I., Cohen, H., Rai, R., Skull, J., Pickering, W. and Regan, L. (2000) Pregnancy outcome is not affected by antiphospholipid antibody status in women referred for in vitro fertilization. Fertil. Steril., **73**, 526-530.

217 Hatasaka, H.H., Branch, D.W., Kutteh, W.H. and Scott, J.R. (1997) Autoantibody screening for infertility: explaining the unexplained? J. Reprod. Immunol., **34**, 137-153.

218 Hornstein, M.D., Davis, O.K., Massey, J.B., Paulson, R.J. and Collins, J.A. (2000) Antiphospholipid antibodies and in vitro fertilization success: a meta-analysis. Fertil. Steril., **73**, 330-333

219 El-Roeiy, A., Gleicher, N., Friberg, J., Confino, E. and Dudkiewicz, A.B. (1987). Correlation between peripheral blood and follicular fluid autoantibodies and impact on in vitro fertilization. Obstet. Gynecol., **70**, 163-170.

220 Gleicher, N., Liu, H.C., Dudkiewicz, A., Rosenwaks, Z., Kaberlein, G., Pratt, D. and Karande. V. (1994) Autoantibody profiles and immunoglobulin levels as predictors of in vitro fertilization success. Am. J. Obstet. Gynecol., **170,** 1145-1149.

221 Kowalik, A, Vichnin, M, Liu, H-C, Branch, W. and Berkeley, A.S. (1997) Midfollicular anticardiolipin and antiphosphatidylserine antibody titres do not correlate with in vitro fertilization outcome Fertil. Steril., **68**, 298-304.

222 Sher, G., Matzner, W., Feinman, M., Maassarani, G., Zouves, C. and Chong, P. (1998) The selective use of heparin/aspirin therapy, alone or in combination with intravenous immunoglobulin G, in the management of antiphospholipid antibody-positive women undergoing in vitro fertilization. Am. J. Reprod. Immunol., **40**, 74-82.

223 Schenk, L.M., Butler, L. and Morris, J.P. (1996) Heparin and aspirin treatment yields higher implantation rates in IVF patients with antiphospholipid antibody seropositivity compared to untreated seronegative patients. American Society of Reproductive Medicine Meeting, O-076.

224 Stern, C., Chamley, L., Norris, H., Hale, L. and Baker, H.W.A (2003) Randomized, double-blind, placebo-controlled trial of heparin and aspirin for women with in vitro fertilization implantation failure and antiphospholipid or antinuclear antibodies. Fertil. Steril., **80**, 376-383.

225 Favre, R., Bettahar, K., Grange, G., Ohl, J., Arbogast, E., Moreau, L. and Dellenbach, P. (1993) Predictive value of transvaginal uterine Doppler assessment in an in vitro fertilization program. Ultrasound. Obstet. Gynecol., **5,** 350-353.

226 Coulam, C.B,. Stern, J.J., Soenksen, D.M., Britten, S. and Bustillo, M. (1995) Comparison of pulsatility indices on the day of oocyte retrieval and embryo transfer. Hum. Reprod., **1**, 82-84.

227 Cacciatore, B. and Tiitinen A. (1996) Does ovarian stimulation affect uterine artery impedance? J. Assist. Reprod. Genet., **13**, 15-18.

228 Wada, I., Hsu, C.C., Williams, G., Macnamee, M.C., and Brindsen, P.R. (1994) The benefits of low-dose aspirin therapy in women with impaired uterine perfusion during assisted conception. Hum. Reprod., **9**, 1954-1957.

229 Battaglia, C., Artini, P.G., Giulini, S., Salvatori, M., Maxia, N., Petraglia, F. and Volpe, A. (1997) Colour Doppler changes and thromboxane production after ovarian stimulation with gonadotrophin-releasing hormone agonist. Hum. Reprod., **11**, 2477-2482.

230 Steer, C.V., Williams, J., Zaidi, J., Campbell, S. and Tan, S.L. (1995) Intra-observer, interobserver, interultrasound transducer and intercycle variation in colour Doppler assessment of uterine artery impedance. Hum. Reprod., **2,** 479-481.

231 Rubinstein, M., Marazzi, A. and Polak de Fried, E. (1999) Low-dose aspirin treatment improves ovarian responsiveness, uterine and ovarian blood flow velocity, implantation, and pregnancy rates in patients undergoing in vitro fertilization: a prospective, randomized, double-blind, placebo-controlled assay. Fertil. Steril., **71**, 825-829.

232 Gilman, A.G., Rall, T.W., Nies, A.S., and Taylor P., (1990) editors. Goodman and Gilman's: The pharmacologic basis of therapeutics (8th ed.), New York: Pergamon. pp 687-733.

233 Vane, J.R. and Botting, R.M. (1992) editors. Aspirin and other salicylates London: Chapman & Hall Medical. pp 35-59.

234 Harper, M.J.K., Kudolo, G.B. and Alecozay, A.A. (1989) Platelet-activating factor (PAF) and blastocyst–endometrial interactions. Prog. Clin. Biol. Res., **294**, 305–315.

235 Harper, M.J.K. (1989) Platelet-activating factor: a paracrine factor in preimplantation stages of reproduction? Biol. Reprod., **40**, 907–913.

236 Van der Weiden, R.M.F., Helmerhorst, F.M. and Keirse, M.J.N.C. (1991) Influence of prostaglandins and platelet activating factor on implantation. Hum. Reprod., **6**, 436-442.

237 Van der Weiden, R.M., Helmerost, F.M. and Keirse, M.J. (1995) Prostanoid excretion before in vitro fertilization relates to the likelihood of pregnancy. Prostaglandins Leukot. Essent. Fatty Acids, **53**, 419-421.

238 Hauth, J., Sibai, B., Caritis, S., VanDorsten, P., Lindheimer, M., Klebanoff, M., MacPherson, C., Landon, M., Paul, R., Miodovnik, M., Meis, P., Dombrowski, M., Thurnau, G., Walsh, S., McNellis, D. and Roberts, J.M. (1998) Maternal serum thromboxane B2 concentrations do not predict improved outcomes in high-risk pregnancies in a low-dose aspirin trial. Am. J. Obstet. Gynecol., **179**, 1193-1199.

239 Deutinger, J., Reinthaller, A. and Bernaschek, G. (1989) Transvaginal pulsed Doppler measurement of blood flow velocity in the ovarian arteries during cycle stimulation and after follicle puncture. Fertil. Steril., **3**, 466-470.

240 Urman, B., Mercan, R., Alatas, C., Balaban, B., Isiklar, A. and Nuhoglu, A. (2000) Low-dose aspirin does not increase implantation rates in patients undergoing intracytoplasmic sperm injection: a prospective randomized study. J. Assist. Reprod. Genet., **17**, 586-590.

241 Kohl-Thomas, B.M., Wincek, T.J., Pliego, J.F., Spiekerman, M., Arizola, D. and Kuehl, T.J. (2002) Absence of improvement by daily low-dose aspirin in ovarian stimulation during ART cycles Fertil. Steril., **78**, S150-S151.

242 Tassa, J.M., Marshburn, P.B., Papadakis, M.A., Loeb, T.A., Matthews, M.M. and Hurst, B.S. (2002) Low-dose aspirin does not improve ovarian stimulation, endometrial response, or pregnancy rates for in vitro fertilization Fertil. Steril., **78**, Supplement 1, S257-S258.

243 Al Salman, F., Felemban, A., Hashim, H., Hassan, S. and Al Bugnah, M. (2002) Does low dose aspirin treatment improve ovarian responsiveness, implantation and pregnancy rate in patients undergoing in vitro fertilization? Middle east fertility society 9th annual scientific meeting, 61.

244 Goswamy, R.K., Williams, G. and Steptoe, P.C. (1988) Decreased uterine perfusion—a cause of infertility. Hum. Reprod., **3**, 955-959.

245 Viinikka, L., Hartikainen-Sorri, A.L., Lumme, R., Hillesmaa, V. and Ylikorkalaa, O. (1993) Low dose aspirin in hypertensive women: effect on pregnancy outcome and prostacyclin-thromboxane balance in mother and newborn. Br. J. Obstet. Gynaecol., **100**, 809-815.

246 Check, J.H., Dietterich, C., Lurie, D., Nazari, A., Chuong, J. (1998) A matched study to determine whether low-dose aspirin without heparin improves pregnancy rates following frozen embryo transfer and/or affects endometrial sonographic parameters. J. Assist, Reprod, Genet., **15**, 579-582.

247 Weckstein, L.N., Jacobson, A., Galen, D., Hampton, K. and Hammel, J. (1997) Low dose aspirin for oocyte donation recipients with a thin endometrium: prospective, randomized study. Fertil. Steril., **68**, 927-930.

248 Kuo, H.C., Hsu, C.C., Wang, S.T. and Huang, K.E. (1997) Aspirin improves uterine blood flow in the peri-implantation period. J. Formos. Med. Assoc., **96**, 253-257.

249 Waldenstrom, U., Hellberg, D. and Nilsson, S. (2004) Low-dose aspirin in a short regimen as standard treatment in in vitro fertilization: a randomized, prospective study. Fertil. Steril., **81**, 1560-1564.

250 Lok, I.H., Yip, S.K., Cheung, L.P., Yin, L.P.H. and Haines, C.J. (2004) Adjuvant low-dose aspirin therapy in poor responders undergoing in vitro fertilization: a prospective, randomized, double-blind, placebo-controlled trial. Fertil. Steril., **81**, 556-561.

251 Pakkila, M., Rasanen, J., Heinonen, S., Tinkanen, H., Tuomivaara, L., Makikallio, K., Hippelainen, M., Tapanainen, J.S. and Martikainen H. (2005) Low-dose aspirin does not improve ovarian responsiveness or pregnancy rate in IVF and ICSI patients: a randomized, placebo-controlled double-blind study. Hum. Reprod., **8**, 2211-2214.

252 Goswamy, R.K. and Steptoe, P.C. (1988) Doppler ultrasound studies of the uterine artery in spontaneous ovarian cycles. Hum. Reprod., **3**, 721-726.

253 Kurjak, A., Kupesic-Urek, S., Schulman, H. and Zalud, I. (1991) Transvaginal color flow Doppler in the assessment of ovarian and uterine blood flow in infertile women. Fertil. Steril., **56**, 870-873.

254 Steer, C.V., Tan, A.L., Mason, B.A. and Campbell, S. (1994) Midluteal-phase vaginal color Doppler assessment of uterine artery impedance in a subfertile population. Fertil. Steril., **61**, 53-58.

255 Sterzik, K., Grab, D., Sasse, V., Hutter, W., Rosenbusch, B. and Terinde, R. (1989) Doppler sonographic findings and their correlation with implantation in an in vitro fertilization program. Fertil. Steril., **52**, 825-828.

256 Coulam, C.B., Bustillo, M., Soenksen, D.M. and Britten, S. (1994) Ultrasonographic predictors of implantation after assisted reproduction. Fertil. Steril., **5,** 1004-1010.

257 Serafini, P., Batzofin, J., Nelson, J. and Olive, D. (1994) Sonographic uterine predictors of pregnancy in women undergoing ovulation induction for assisted reproductive treatments. Fertil. Steril., **62**, 815-822.

258 Tekay, A., Martikainen, H. and Jouppila, P. (1995) Blood flow changes in uterine and ovarian vasculature, and predictive value of transvaginal pulsed colour Doppler ultrasonography in an in-vitro fertilization programme. Hum. Reprod., **10**, 688-693.

259 Zaidi, J., Barber, J., Kyei-Mensah, A., Bekir, J., Campbell, S. and Tan, S.L. (1996) Relationship of ovarian stromal blood flow at the baseline ultrasound scan to subsequent follicular response in an in vitro fertilization program. Obstet. Gynecol., **88**, 779-784

260 Zaidi, J., Pittrof, R., Shaker, A., Kyei-Mensah, A., Campbell, S. and Tan, S.L. (1996) Assessment of uterine artery blood flow on the day of human chorionic gonadotropin administration by transvaginal color Doppler ultrasound in an in vitro fertilization program. Fertil. Steril., **2,** 377-381.

261 Steer, C.V., Campbell, S., Pampiglione, J.S., Kingsland, C.R., Mason, B.A. and Collins, W.P. (1990) Transvaginal colour flow imaging of the uterine arteries during the ovarian and menstrual cycles. Hum. Reprod., **4**, 391-395.

262 Rosenfeld, C.R., Morriss, F.H. Jr., Battaglia, F.C., Makowski, E.L. and Meschia, G. (1976) Effect of estradiol-17beta on blood flow to reproductive and nonreproductive tissues in pregnant ewes. Am. J. Obstet. Gynecol., **124**, 618-629.

263 Fraser, I.S., McCarron, G., Hutton, B., Macey, D., (1987) Endometrial blood flow measured by xenon 133 clearance in women with normal menstrual cycles and dysfunctional uterine bleeding. Am. J. Obstet. Gynecol., **156**, 158-166.

264 Bloechle, M., Schreiner, T., Kuchler, I., Schurenkamper, P. and Lisse, K. (1997) Colour Doppler assessment of ascendent uterine artery perfusion in an in-vitro fertilization-embryo transfer programme after pituitary desensitization and ovarian stimulation with human recombinant follicle stimulating hormone. Hum Reprod., **8,** 1772-1777.

265 Magness, R.R., Parker, C.R. and Rosenfeld, C.R. (1993) Systemic and uterine responses to chronic infusion of estradiol-17. Am. J. Physiol., **265**, E690–E698.

266 de Ziegler, D., Bessis, R. and Frydman, R. (1991) Vascular resistance of uterine arteries: physiological effects of estradiol and progesterone. Fertil. Steril., **55**, 775-779.

267 Hillard, T.C., Bourne, T.H., Whitehead, M.I., Crayford, T.B,. Collins, W.P. and Campbell, S. (1992) Differential effects of transdermal estradiol and sequential progestogens on impedance to flow within the uterine arteries of postmenopausal women. Fertil. Steril., **5**, 959-963.

268 Marsh, M.S., Bourne, T.H., Whitehead, M.I., Collins, W.P. and Campbell, S. (1994) The temporal effect of progestogen on uterine artery pulsatility index in postmenopausal women receiving sequential hormone replacement therapy. Fertil. Steril., **4**, 771-774.

269 Zaidi, J., Jurkovic, D., Campbell, S., Pittrof, R., McGregor, A. and Tan, S.L. (1995) Description of circadian rhythm in uterine artery blood flow during the peri-ovulatory period. Hum. Reprod., **7**, 1642-1646.

270 Tinkanen, H., Kujansuu, E. and Laippala, P. (1994) Vascular resistance in uterine and ovarian arteries: its association with infertility and the prognosis of infertility. Eur. J. Obstet. Gynecol. Reprod. Biol., **2**, 111-115.

271 Strohmer, H., Herczeg, C., Plockinger, B., Kemeter, P. and Feichtinger, W. (1991) Prognostic appraisal of success and failure in an in vitro fertilization program by transvaginal Doppler ultrasound at the time of ovulation induction. Ultrasound Obstet. Gynecol., **4,** 272-274.

272 Levi-Setti, P.E., Rognoni, G., Bozzo, M., Ragusa, G., Sulpizio, P., Ferrazzi, E. and Pardi, G. (1995) Color-Doppler velocimetry of uterine arteries in pregnant and nonpregnant patients during multiovulation induction for IVF. J. Assist. Reprod. Genet. , **7**, 413-417.

273 Cacciatore, B., Simberg, N., Fusaro, P. and Tiitinen, A. (1996) Transvaginal Doppler study of uterine artery blood flow in in vitro fertilization-embryo transfer cycles. Fertil Steril., **66**, 130-134.

274 Yuval, Y., Lipitz, S., Dor, J. and Achiron, R. (1999) The relationships between endometrial thickness, and blood flow and pregnancy rates in in-vitro fertilization. Hum. Reprod., **14,** 1067-1071.

275 Bassil, S., Magritte, J.P., Roth, J., Nisolle, M., Donnez, J. and Gordts, S. (1995) Uterine vascularity during stimulation and its correlation with implantation in in-vitro fertilization. Hum. Reprod., **6**, 1497-1501.

276 Fugino, Y., Ito, F., Matuoka, I., Kojima, T., Koh, B. and Ogita, S. (1993) Pulsatility index of uterine artery in pregnant and non-pregnant women. Hum. Reprod. **7**, 1126-1128.

277 Bustillo, M., Krysa, L.W. and Coulman, C.B. (1995) Uterine receptivity in an oocyte donation programme. Hum. Reprod., **10**, 442-445

278 Engmann , L., Sladkevicius, P., Agrawal, R., Bekir, J.S., Campbell, S. and Tan, S.L. (1999) Value of stromal blood flow velocity measurement after pituitary suppression in the prediction of ovarian responsiveness and outcome of in vitro fertilization treatment. Fertil. Steril., **71**, 22-29.

279 Salle, B., Bied-Damon, V., Benchaib, M., Desperes, S., Gaucherand, P. and Rudigoz, R.C. (1998) Preliminary report of an ultrasonography and colour Doppler uterine score to predict uterine receptivity in an in-vitro fertilization programme. Hum. Reprod., **6**, 1669-1673.

280 Aytoz, A., Ubaldi, F., Tournaye, H., Nagy, Z.P., Van Steirteghem, A. and Devroey, P. (1997) The predictive value of uterine artery blood flow measurements for uterine receptivity in an intracytoplasmic sperm injection program. Fertil. Steril.,. **5**, 935-937.

281 Schild, R.L., Indefrei, D., Eschweiler, S., Van der Ven, H., Fimmers, R. and Hansmann, M. (1999) Three dimensional endometrial volume calculation and pregnancy rate in an in-vitro fertilization program. Hum. Reprod., **14,** 1255–1258.

282 Schild, R.L., Holthaus, S., d'Alquen, J., Fimmers, R., Dorn, C., van Der Ven, H. and Hansmann, M. (2000) Quantitative assessment of subendometrial blood flow by three-dimensional-ultrasound is an important predictive factor of implantation in an in-vitro fertilization programme. Hum. Reprod., **1**, 89-94.

283 Schild, R.L., Knobloch, C., Dorn, C., Fimmers, R. and Manfred, H.V. (2001) Endometrial receptivity in an in vitro fertilization program as assessed by spiral artery blood flow, endometrial thickness, endometrial volume, and uterine artery blood flow. Fertil. Steril., **2**, 361-366

284 Basir, G.S., Lam, P.W.T., Chau, M.T., Ng, E.H.Y., O, W.S. and Ho, P.C. (2001) Hemodynamic evaluation of tubal and male factors of infertility in natural and ovarian stimulation cycles. J. Assist. Reprod. Genet., **3**,125-128.

285 Guanes, P.P., Remohi, J., Gallardo, E., Valbuena, D., Simon, C. and Pellicer, (1996) A. Age does not affect uterine resistance to vascular flow in patients undergoing oocyte donation. Fertil Steril., **2**, 265-270.

286 Check, J.H., Dietterich, C., Lurie, D. and Nazari, A. (2000) No evidence of increased uterine vascular impedance with patient ageing following IVF. Hum. Reprod., **8**, 1679-1684.

287 Kurjak, A. and Kupesic, S. (1995) Ovarian senescence and its significance on uterine and ovarian perfusion. Fertil. Steril., **64**, 532-537

288 Friedler, S., Schenker, J.G., Herman, A. and Lewin, A. (1996) The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: a critical review. Hum. Reprod. Update, **2**, 323-335.

289 Dickey, R.P. (1997) Doppler ultrasound investigation of uterine and ovarian blood flow in infertility and early pregnancy. Hum. Reprod. Update., **3,** 467-503.

290 Tekay, A., Martikainen, H. and Jouppila, P. (1996) The clinical value of transvaginal colour Doppler ultrasound in assisted reproductive technology procedures. Hum. Reprod., **11**, 1589-1591.

291 Yang, J.H., Wu, M.Y., Chen, C.D., Jiang, M.C., Ho, H.N. and Yang , Y.S. (1999) Association of endometrial blood flow as determined by a modified color Doppler technique with subsequent outcome of in-vitro fertilization. Hum. Reprod., **14** 1606-1610.

292 Applebaum, M. (1995) The uterine biophysical profile. Ultrasound Obstet. Gynecol., **5** 67-68.

293 Zaidi, J., Campbell, S., Pittrof, R. and Tan, S.L. (1995) Endometrial thickness, morphology, vascular penetration and velocimetry in predicting implantation in an in vitro fertilization program. Ultrasound Obstet. Gynecol., **6**, 191-198.

294 Kupesic, S., Bekavac, I., Bjelos, D. and Kurjak , A. (2001) Assessment of endometrial receptivity by transvaginal color Doppler and three-dimensional power Doppler ultrasonography in patients undergoing in vitro fertilization procedures. J. Ultrasound Med., **20**, 125-134.

295 Taylor, G.A., Ecklund, K. and Dunning, P.S. (1996) Renal cortical perfusion in rabbits: visualization with colour amplitude imaging and an experimental microbubble-based US contrast agent. Radiology, **201**, 125-129

296 Jain, S.P., Fan, P.H., Philpot, E.F., Nanda, N.C., Aggarwal, K.K., Moos, S. and Yoganathan, A.P. (1991) Influence of various instrument settings on the flow information derived from the power mode. Ultrasound Med. Biol., **1**, 49-54.

297 Huber, S., Delorme, S., Knopp, M.V., Junkermann, H., Zuna, I., von Fournier, D. and van Kaick, G. (1994) Breast tumors: computer-assisted quantitative assessment with color Doppler US. Radiology., **3**, 797-801.

298 Kedar, R.P., Cosgrove, D.O., Bamber, J.C. and Bell, D.S. (1995) Automated quantification of color Doppler signals: a preliminary study in breast tumors. Radiology. **1**, 39-43.

299 Wu, M.H., Tang, H.H., Hsu, C.C., Wang, S.T. and Huang, K.E. (1998) The role of three-dimensional ultrasonographic images in ovarian measurement. Fertil. Steril., **6,** 1152-1155.

300 McCarthy, S., Scott, G., Majumdar, S., Shapiro, B., Thompson, S., Lange, R. and Gore, J. (1989) Uterine junctional zone: MR study of water content and relaxation properties. Radiology., **1,** 241-243.

301 Scoutt, L.M., Flyn, S.D., Luthringer, D.J., McCauley, T.R. and McCarthy, S.M. (1991) Junctional zone of the uterus: correlation of MR imaging and histologic examination of hysterectomy specimens. Radiology., **179,** 403-407.

302 Tetlow, R.L., Richmond, I., Manton, D.J., Greenman, J., Turnbull, L.W. and Killick, S.R. (1999) Histological analysis of the uterine junctional zone as seen by transvaginal ultrasound. Ultrasound Obstet. Gynecol., **14,** 188-193.

303 Bourne, T.H., Hagstrom, H.G., Granberg, S., Josefsson, B., Hahlin, M., Hellberg. P., Hamberger, L. and Collins, W.P. (1996) Ultrasound studies of vascular and morphological changes in the human uterus after a positive self-test for the urinary luteinizing hormone surge. Hum Reprod., **2**, 369-375.

304 Contart, P., Baruffi, R.L., Coelho, J., Mauri, A.L., Petersen, C. and Franco, J.G. Jr. (2000) Power Doppler endometrial evaluation as a method for the prognosis of embryo implantation in an ICSI program. J. Assist. Reprod. Genet., **17,** 329-334.

305 Noe, M., Kunz, G., Herbertz, M., Mall, G. and Leyendecker, G. (1999) The cyclic pattern of the immunocytochemical expression of oestrogen and progesterone receptors in human myometrial and endometrial layers: characterization of the endometrial-subendometrial unit. Hum. Reprod., **14,** 190-197.

306 Lesny, P., Killick, S.R., Tetlow, R.L., Manton, D.J., Robinson, J. and Maguiness, S.D. (1999) Ultrasound evaluation of the uterine zonal anatomy during in-vitro fertilization and embryo transfer. Hum. Reprod., **14,** 1593-1598.

307 Chien, L., Au, H., Chen, P., Xiao, J. and Tzeng, C. (2002) Assessment of uterine receptivity by the endometrial-subendometrial blood flow distribution pattern in women undergoing in vitro fertilization-embryo transfer. Ferti. Steril., **2**, 245-251.

308 Jinno, M., Ozaki, T., Iwashita, M., Nakamura, Y., Kudo, A. and Hirano, H. (2001) Measurement of endometrial tissue blood flow: a novel way to assess uterine receptivity for implantation. Fertil. Steril., **6**, 1168-1174.

309 Basir, G.S, Lam, T.P., O W, Chau, M.T., Ng, E.H. and Ho, P.C. (2002) Cycle-to-cycle variation in utero-ovarian hemodynamic indices in ovarian stimulation and natural cycles of the same women and its effect on the outcome of assisted reproduction treatment. Fertil. Steril., **5**,1055-1060.

310 Long, M.G., Boultbee, J.E., Hanson, M.E. and Begent, R.H.J. (1989) Doppler time velocity waveform studies of the uterine artery and uterus. Br. J. Obstet. Gynecol., **96**, 588-593.

311 Baruffi, R.L., Contart, P., Mauri, A.L., Petersen, C., Felipe, V., Garbellini, E., Franco, J.G.A. (2002) uterine ultrasonographic scoring system as a method for the prognosis of embryo implantation. J. Assist. Reprod. Genet., **3**, 99-102.

312 Tekay, A. and Joupilla, P. (1996) Intraobserver reproducibility of transvaginal Doppler measurements in uterine and intraovarian arteries in regularly menstruating women. Ultrasound Obstet. Gynecol., **7**, 129-134.

313 Weiner, Z., Thaler, I., Levron, J., Lewit, N. and Itskovitz-Eldor, J. (1993) Assessment of ovarian and uterine blood flow by transvaginal color Doppler in ovarian-stimulated women: correlation with the number of follicles and steroid hormone levels. Fertil. Steril., **59**, 743-749.

314 Strigini, F.A., Scida, P.A., Parri, C., Visconti, A., Susini, S. and Genazzani, A.R. (1995) Modifications in uterine and intraovarian artery impedance in cycles of treatment with exogenous gonadotropins: effects of luteal phase support. Fertil. Steril., **64**, 76-80.

315 Glock, J. L. and Brumsted, J. R. (1995) Colour flow pulsed Doppler ultrasound in diagnosing luteal phase defect. Fertil. Steril., **64**, 500-504.

316 Zaidi, J., Campbell, S., Pittrof, R., Kyei-Mensah, A., Shaker, A., Jacobs, H.S. and Tan, S.L. (1995) Ovarian stromal blood flow in women with polycystic ovaries - a possible new marker for diagnosis? Hum. Reprod., **10**, 1992-1996.

317 Oyesanya, O.A., Parsons, J.H., Collins, W.P. and Campbell, S. (1996) Prediction of oocyte recovery rate by transvaginal ultrasonography and color Doppler imaging before human chorionic gonadotropin administration in in vitro fertilization cycles. Fertil. Steril., **65**, 806-809.

318 Barber, R.J., McSweeney, M.B. and Gill, R.W. (1988) Transvaginal pulsed Doppler ultrasound assessment of blood flow to the corpus luteum in IVF patients following embryo transfer. Br. J. Obstet. Gynaecol., **95,** 1226-1230.

319 Nargund, G., Doyle, P.E., Bourne, T.H., Parsons, J.H., Cheng, W.C., Campbell, S. and Collins, W.P. (1996) Ultrasound derived indices of follicular blood flow before HCG administration and prediction of oocyte recovery and preimplantation embryo quality. Hum. Reprod., **11**, 2512–2517.

320 Chui, D.K., Pugh, N.D., Walker, S.M., Gregory, L., Shaw, R.W. (1997) Follicular vascularity--the predictive value of transvaginal power Doppler ultrasonography in an in-vitro fertilization programme: a preliminary study. Hum. Reprod., **1,** 191-196.

321 Bhal, P.S., Pugh, N.D., Chui, D.K., Gregory, L., Walker, S.M. and Shaw, R.W. (1999) The use of transvaginal power Doppler ultrasonography to evaluate the relationship between perifollicular vascularity and outcome in in-vitro fertilization treatment cycles. Hum. Reprod., **14**, 939-945.

322 Coulam, C.B., Goodman, C. and Rinehart, J.S. (1999) Colour Doppler indices offollicular blood flow aw as prediciors of pregnancy after in vitro fertilization and embryo transfer. Hum. Reprod., **14**, 1979-1982.

323 Balakier, H. and Stronell, R.D. (1994) Colour Doppler assessment of folliculogenesis in in vitro fertilization patients. Fertil. Steril., **62,** 1211-1216.

324 Nargund, G., Bourne, T., Doyle, P., Parsons, J., Cheng, W., Campbell, S. and Collins, (1996) W. Associations between ultrasound indices of follicular blood flow, oocyte recovery and preimplantation embryo quality. Hum. Reprod., **1**,109-113.

325 Risau, W. (1997) Mechanisms of angiogenesis. Nature (London)., **368**, 671-674.

326 Torry, D.S. and Torry, R.J. (1997) Angiogenesis and the expression of vascular endothelial growth factor in endometrium and placenta. Am. J. Reprod. Immunol., **37**, 21-29.

327 Rogers, P.A.W. and Gargett, C.E. (1999) Endometrial angiogenesis. Angiogenesis., **2**, 287-294.

328 Hanahan, D. and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell., **86,** 353-364.

329 Ferrara, N. (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am. J. Physiol. Cell Physiol., **280,** C1358-C1366.

330 Roberts, W.G. and Palade, G.E. (1995) Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. J. Cell Sci., **108**, 2369–2379.

331 Papapetropoulos, A., Garcia-Gardena. G., Madri. J.A. and Sessa. W.C. (1997) Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J. Clin. Invest., **100**, 3131-3139.

332 Wheeler-Jones, C., Abu-Ghazaleh, R., Cospedal, R., Houliston, R.A., Martin, J. and Zachary, I. (1997) Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. FEBS Lett., **1**, 28-32.

333 Ferrara, N. and Davis-Smyth, T. (1997) The biology of vascular endothelial growth factor. Endocr. Rev., **18**, 4-25

334 Fujisawa, H. and Kitsukawa, T. (1998) Receptors for collapsing/semaphorins. Curr. Opin. Neurobiol., **8**, 587-592.

335 Soker, S. (2001) Neuropilin in the midst of cell migration and retraction. Int. J. Biochem. Cell. Biol., **33**, 433-437.

336 Weston, G. and Rogers, P.A. (2000) Endometrial angiogenesis. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol., **6**, 919-936.

337 Iruela-Arispe, M.L., Porter, P., Bornstein, P. and Sage, E.H.(1996) Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium. J. Clin. Invest., **2**, 403-412.

338 Sheibani, N., Newman, P.J. and Frazier, W.A. (1997) Thrombospondin-1, a natural inhibitor of angiogenesis, regulates platelet–endothelial cell adhesion molecule-1 expression and endothelial cell morphogenesis. Mol. Biol. Cell., **8,** 1329–1341.

339 O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R. and Folkman, J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, **2**, 277-285.

340 Mandriota, S.J., Menoud, P.A. and Pepper, M.S. (1996) Transforming growth factor beta 1 down-regulates vascular endothelial growth factor receptor 2/flk-1 expression in vascular endothelial cells. J. Biol. Chem., **19**, 11500-11505.

341 Van Blerkom, J. (2000) Intrafollicular influences on human oocyte developmental competence: perifollicular vascularity, oocyte metabolism and mitochondrial function. Hum. Reprod., **15,** Suppl 2, 173-188

342 Huey, S., Abuhamad, A., Barosso, G., Hsu, M.I., Kolm, P. and Mayer, J. (1999) Perifollicular blood flow Doppler indices, but not follicular pO_2, pCO_2 or pH, predict oocyte developmental competence in IVF. Fertil. Steril., **72**, 707–712.

343 Barroso, G., Barrionuevo, M., Rao, P., Graham, L., Danforth, D., Huey, S., Abuhamad, A. and Oehninger, S. (1999) Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil. Steril., **6**, 1024-1026.

344 Luo, H., Kimura, K., Aoki, M. and Hirako, M. (2002) Effect of vascular endothelial growth factor on maturation, fertilization and developmental competence of bovine oocytes. J. Vet. Med. Sci., **9**, 803-806.

345 Lee, A., Christenson, L.K., Patton, P.E, Burry, K.A. and Stouffer, R.L. (1997) Vascular endothelial growth factor production by human luteinized granulosa cells in vitro. Hum. Reprod., **12**, 2756-2761.

346 Moncayo, H.E., Penz-Koza, A., Marth, C., Gastl, G., Herold, M. and Moncayo, R. (1998) Vascular endothelial growth factor in serum and in the follicular fluid of patients undergoing hormonal stimulation for in-vitro fertilization. Hum. Reprod., **12**, 3310-3314.

347 Manau, D., Balasch, J., Jimenez, W., Fabregues, F., Civico, S., Casamitjana, R., Creus, M. and Vanrell, J.A. (2000) Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. Hum. Reprod., **6**, 1295-1299.

348 Doldi, N., Bassan, M., Fusi, F.M. and Ferrari, A. (1997) In controlled ovarian hyperstimulation, steroid production, oocyte retrieval, and pregnancy rate correlate with gene expression of vascular endothelial growth factor. J. Assist. Reprod. Genet., **10,** 589-592.

349 Friedman, C.I., Seifer, D.B., Kennard, E.A., Arbogast, L., Alak, B. and Danforth, D.R. (1998) Elevated level of follicular fluid vascular endothelial growth factor is a marker of diminished pregnancy potential. Fertil. Steril., **70**, 836-839.

350 Younis, J.S., Haddad, S. and Matilsky, M. (1998) Premature luteinization, could it be an early manifestation of low ovarian reserve, and M. Ben-Am. Fertil. Steril., **69**, 461-465.

351 Christenson, L,K. and Stouffer, R,L. (1997) Follide-stimulatmg hormone and luteinizing hormone/chorionic Gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. J. Clin. Endocrinol. Metab., **82**, 2135-2142.

352 Artini, P.G., Monti, M., Fasciani, A., Tartaglia, M.L., D'Ambrogio, G. and Genazzani, A.R. (1998) Correlation between the amount of follicle-stimulating hormone administered and plasma and follicular fluid vascular endothelial growth factor concentrations in women undergoing in vitro fertilization. Gynecol. Endocrinol., **4,** 243-237.

353 Enskog, A., Nilsson, L. and Brannstrom, M. (2001) Plasma levels of free vascular endothelial growth factor(165) (VEGF(165)) are not elevated during gonadotropin stimulation in in vitro fertilization (IVF) patients developing ovarian hyperstimulation syndrome (OHSS): results of a prospective cohort study with matched controls. Eur. J. Obstet. Gynecol. Reprod. Biol., **2,** 196-201.

354 Licht, P., Neuwinger, J., Fischer, O., Siebzehnrubl, E., Wildt, L. (2001) Peripheral levels of vascular endothelial growth factor (VEGF) are higher in gonadotropin stimulated as compared to natural ovarian cycles. Exp. Clin. Endocrinol. Diabetes., **6,** 345-349.

355 Anasti, J.N., Kalantaridou, S.N., Kimzey, L.M., George, M. and Nelson, L.M. (1998) Human follicle fluid vascular endothelial growth factor concentrations are correlated with luteinization in spontaneously developing follicles. Hum. Reprod., **5**, 1144-1147.

356 Doldi, N., Destefani, A., Gessi, A., Grossi, D. and Ferrari, A. (1999) Human albumin enhances expression of vascular endothelial growth factor in cultured human luteinizing granulosa cells: importance in ovarian hyperstimulation syndrome. Hum Reprod., **5**, 1157-1159.

357 Van Blerkom, J. (1998) Epigenetic influences on oocyte developmental competence: perifollicular vascularity and intrafollicular oxygen. J. Assist. Reprod. Genet., **15**, 226-233.

358 Malamitsi-Puchner, A., Sarandakou, A. M., Stavroula, Baka, G., Tziotis, J., Rizos, D., Hassiakos, D. and Creatsas, G. M.D. (2001) Concentrations of angiogenic factors in follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity and fertilization. Fertil. Steril., **1**, 98-101.

359 Shweiki, D., Itin, A., Soffer, D. and E. Keshet. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature., **359,** 843-845.

360 Gerber, H.P., Condorelli, F., Park, J. and Ferrara, N. (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. J. Biol. Chem., **272**, 23659-23667.

361 Friedman, C.I., Danforth, D.R., Herbosa-Encarnacion, C., Arbogast, L., Alak, B.M. and Seifer, D.B. (1997) Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. Fertil. Steril., **68**, 607-612.

362 Battaglia, C., Genazzani, A.D., Regnani, G., Primavera, M.R., Petraglia, F. and Volpe, A. (2000) Perifollicular Doppler flow and follicular fluid vascular endothelial growth factor concentrations in poor responders. Fertil. Steril., **74**, 809-812

363 Quintana, R., Kopcow, L., Marconi, G., Sueldo, C., Speranza, G. and Barañao, R.I. (2001) Relationship of ovarian stimulation response with vascular endothelial growth factor and degree of granulosa cell apoptosis. Hum. Reprod., **9**, 1814-1818.

364 Benifla, J.L., Bringuier, A.F., Sifer, C., Porcher, R., Madelenat P., Feldmann G. (2001) Vascular endothelial growth factor, platelet endothelial cell adhesion molecule-1 and vascular cell adhesion molecule-1 in the follicular fluid of patients undergoing IVF. Hum. Reprod., **7**, 1376-1381.

365 He, Y., Smith, S.K., Day, K.A., Clark, D.E., Licence, D.R. and Charnock-Jones, D.S. (1999) Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. Mol. Endocrinol., **4**,537-545.

366 Neulen, J., Wenzel, D., Hornig, C., Wunsch, E., Weissenborn, U., Grunwald, K., Buttner, R. and Weich, H. (2001) Poor responder–high responder: the importance of soluble vascular endothelial growth factor receptor 1 in ovarian stimulation protocols. Hum. Reprod., **16**, 621-626.

367 Bergh, P.A. and Navot, D. (1992) The impact of embryonic development and endometrial maturity on the timing of implantation. Fertil. Steril., **58**, 537-542.

368 Lessey, B.A. (2000) Endometrial receptivity and the window of implantation. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol.,**14**, 775-788.

369 Aplin, J.D. (2000) The cell biological basis of human implantation Bailliere's Clinical Obstet. Gynaecol., **5**, 757-764.

370 Formigli, L., Formigli, G. and Roccio, C. (1987) Donation of fertilized uterine ova to infertile women. Fertil. Steril., **47**, 162–165.

371 Psychoyos, A. (1986) Uterine receptivity for nidation. Ann. N.Y. Acad. Sci., **476**, 36-42.

372 Lenton, E.A., Neal, L.M. and Sulaiman, R. (1982) Plasma concentrations of human chorionic gonadotropin from the time of implantation until the second week of pregnancy. Fertil. Steril., **37**, 773-778.

373 Nikas, G., Drakakis, P., Loutradis, D., Mara-Skoufari, C., Koumantakis, E. and Michalas, S. (1995) Uterine pinopodes as markers of the `nidation window' in cycling women receiving exogenous oestradiol and progesterone. Hum. Reprod., **10**, 1208-1213.

374 Kolb, B.A. and Paulson, R.J. (1997) The luteal phase of cycles utilizing controlled ovarian hyperstimulation and the possible impact of this hyperstimulation on embryo implantation. Am. J. Obstet. Gynecol., **176**, 1262–1269.

375 Nikas, G. and Psychoyos, A. (1997) Uterine pinopodes in periimplantation human endometrium. Clinical relevance. Ann. N. Y. Acad. Sci., **816**, 129-142.

376 Noyes, R.W., Hertig, A.T. and Rock, J. (1975) Dating the endometrial biopsy. Am. J. Obstet. Gynecol., **2**, 262-263.

377 Csemiczky, G., Wramsby, H., Johannisson, E. and Landgren, B.M. (1998) Importance of endometrial quality in women with tubal infertility during a natural menstrual cycle for the outcome of IVF treatment. J. Assist. Reprod. Genet., **2**, 55-61.

378 Li, T.C., Warren, M.A. and Cooke, I.D. (1993) Is the measurement of estradiol/progesterone ratios in the peri-implantation period of any value in predicting endometrial development? Hum. Reprod., **8**, 374–378.

379 Ben-Nun, I., Less, A., Kaneti, H., Ghetler, Y., Shilon, M., Shulman, A., Bahary, C., Beyth, Y. and Siegal, A. (1992) Lack of correlation between hormonal blood levels and endometrial maturation in agonadal women with repeat implantation failure following embryo transfer from donated eggs. J. Assist. Reprod. Genet., **2**, 102-105.

380 Fleischer, A.C., Pittaway, D.E., Beard, L.A., Thieme, G.A., Bundy, A.L., James, A.E. Jr. and Wentz, A.C. (1984) Sonographic depiction of endometrial changes occurring with ovulation induction. J. Ultrasound Med., **8**, 341-346.

381 Smith, B., Porter, R., Ahuja, K. and Craft, I. (1984) Ultrasonic assessment of endometrial changes in stimulated cycles in an in vitro fertilization and embryo transfer program. J. In Vitro Fertil. Embryo Transf., **1**, 233–238.

382 Gonen, Y. and Casper, R.F. (1990) Prediction of implantation by the sonographic appearance of the endometrium during controlled ovarian stimulation for in vitro fertilization (IVF). J. In Vitro Fertil. Embryo Transf., **7**, 146-152.

383 Sher, G., Herbert, C., Maassarani, G. and Jacobs, M.H. (1991) Assessment of the late proliferative phase endometrium by ultrasonography in patients undergoing in-vitro fertilization and embryo transfer (IVF/ET). Hum. Reprod., **6**, 232–237.

384 Forrest, T.S., Elyaderani, M.K., Muilenburg, M.I., Bewtra, C., Kable, W.T. and Sullivan, P. (1988) Cyclic endometrial changes: US assessment with histologic correlation. Radiology, **167**, 233–237.

385 Fleischer, A.C., Kalemeris, G., Machin, J.E., Entman, S.S. and James, E.A. (1986) Sonographic depiction of normal and abnormal endometrium with histopathologic correlation. J. Ultrasound Med., **5**, 445–452.

386 Grunfeld, L., Walker, B., Bergh, P.A., Sandler, B., Hofmann, G. and Navot, D. (1991) High-resolution endovaginal ultrasonography of the endometrium: a noninvasive test for endometrial adequacy. Obstet. Gynecol., **2**, 200-204.

387 Fanchin, R., Righini, C., Olivennes, F., Taieb, J., De Ziegler, D. and Frydman, R. (1999) Computerized assessment of endometrial echogenicity: clues to the endometrial effects of premature progesterone elevation. Fertil. Steril., **71**, 174-181.

388 Glissant, A., De Mouzon, J. and Frydman, R. (1985) Ultrasound study of the endometrium during in vitro fertilization cycles. Fertil. Steril., **44**, 786-790.

389 Gonen, Y., Casper, R.F., Jacobson, W. and Blankier, J. (1989) Endometrial thickness and growth during ovarian stimulation: a possible predictor of implantation in in vitro fertilization. Fertil. Steril., **52**, 446-450.

390 Dickey, R.P., Olar, T.T., Curole, D.N., Taylor, S.N. and Rye, P.H. (1992) Endometrial pattern and thickness associated with pregnancy outcome after assisted reproduction technologies. Hum. Reprod., **7**, 418-421.

391 Abdalla, H.I., Brooks, A.A., Johnson, M.R., Kirkland, A., Thomas, A. and Studd, J.W. (1994) Endometrial thickness: a predictor of implantation in ovum recipients?. Hum. Reprod., **9**, 363-365.

392 Noyes, N., Liu, H.C., Sultan, K., Schattman, G. and Rosenwaks, Z. (1995) Endometrial thickness appears to be a significant factor in embryo implantation in in-vitro fertilization. Hum. Reprod., **10**, 919-922.

393 Check, J.H., Nowroozi, K., Choe, L. and Dietterich, C. (1991) Influence of endometrial thickness and echogenic patterns on pregnancy rates during in vitro fertilization. Fertil. Steril., **56**, 1173-1175.

394 Remohi, J., Ardiles, G., Garcia-Velasco, J.A., Gaitan, P., Simon, C. and Pellicer, A. (1997) Endometrial thickness and serum oestradiol concentrations as predictors of outcome in oocyte donation. Hum. Reprod., **12**, 2271-2276.

395 Fleischer, A.C., Herbert, C.M., Sacks, G.A., Wentz, A.C., Entman, S.S. and James, A.E. Jr. (1986) Sonography of the endometrium during conception and nonconception cycles of in vitro fertilization and embryo transfer. Fertil. Steril., **3,** 442-447.

396 Welker, B.G., Gembruch, U., Diedrich, K., Al-Hasani, S. and Krebs, D. (1989) Transvaginal sonography of the endometrium during ovum pickup in stimulated cycles for in vitro fertilization. J. Ultrasound Med., **8,** 549-553.

397 Khalifa, E., Brzyski, R.G., Oehninger, S., Acosta, A.A. and Muasher, S.J. (1992) Sonographic appearance of the endometrium: the predictive value for the outcome of in-vitro fertilization in stimulated cycles. Hum. Reprod., **7**, 677–680.

398 Oliveira, J.B., Baruffi, R.L., Mauri, A.L., Petersen, C.G., Campos, M.S. and Franco, J.G. Jr., (1993) Endometrial ultrasonography as predictor of pregnancy in an in-vitro fertilization programme. Hum. Reprod., **8**, 1312-1315.

399 Sundström, P. (1998) Establishment of a successful pregnancy following in-vitro fertilization with an endometrial thickness of no more than 4 mm. Hum. Reprod., **13**, 1550-1552.

400 Sharara, F. and McClamrock, H. (1999) The effect of aging on ovarian volume measurements in infertile women.Obstet. Gynecol., **94**, 57-60.

401 Leibovitz, Z., Grinin, V., Rabia, R., Degani, S., Shapiro, I., Tal, J., Eibschitz, I., Harari, O., Paltieli, Y., Aharoni, A., Zeevi, J. and Ohel, G. (1999) Assessment of endometrial receptivity for gestation in patients undergoing in vitro fertilization, using endometrial thickness and the endometrium-myometrium relative echogenicity coefficient. Ultrasound Obstet. Gynecol., **14**, 194-199.

402 Rabinowitz, R., Laufer, N. and Lewin, A. (1986) The value of ultrasonographic endometrial measurement in the prediction of pregnancy following in-vitro fertilization. Fertil. Steril., **45**, 824-828.

403 Oliveira, J.B.A., Baruffi, R.L.R. and Mauri, A.L. (1997) Endometrial ultrasonography as a predictor of pregnancy in an in-vitro fertilization program after ovarian stimulation and gonadotropin-releasing hormone and gonadotropins. Hum. Reprod., **12**., 2515-2518.

404 Ueno, J., Oehninger, S., Brzyski, R.G., Acosta, A.A., Philput, C.B. and Muasher, S.J. (1991) Ultrasonographic appearance of the endometrium in natural and stimulated in-vitro fertilization cycles and its correlation with outcome. Hum. Reprod., **6**, 901-904.

405 Bohrer, M.K., Hock, D.L., Rhoads, G.G. and Kemmann, E. (1996) Sonographic assessment of endometrial pattern and thickness in patients treated with human menopausal gonadotropins. Fertil. Steril., **66**, 244-247.

406 Schwartz, L.B., Chiu, A.S., Courtney, M., Krey, L. and Schmidt-Sarosi, C. (1997) The embryo versus endometrium controversy revisited as it relates to predicting pregnancy outcome in in-vitro fertilization-embryo transfer cycles. Hum. Reprod., **12**, 45-50.

407 Turnbull, L.W., Rice, C.F., Horsman, A., Robinson, J. and Killick, S.R. (1994) Magnetic resonance imaging and transvaginal ultrasound of the uterus prior to embryo transfer. Hum. Reprod., **9**, 2438-2443.

408 Strohmer, H., Obruca, A., Radnevk, M. and Feichtinger, W. (1994) Relationship of the individual uterine size and the endometrial thickness in stimulated cycles. Fertil. Steril. **61**, 972-975.

409 Al-Shawaf, T., Yang, D., Al-Magid, Y., Seaton, A., Iketubosin, F. and Craft, I. (1993) Ultrasonic monitoring during replacement of frozen/thawed embryos in natural and hormone replacement cycles. Hum. Reprod., **12,** 2068-2074.

410 Friedler, S., Tanos, V., Reubinoff, B.E., Lewin, A. and Schenker, G.J. (1994) The role of endometrial thickness in conception and nonconception cryopreserved–thawed embryo transfer during natural cycles. Presented at the ESHRE Workshop on Regulation of Fertility, Jerusalem, Israel, 27-31.

411 Alam, V., Bernardini, L., Gonzales, J., Asch, R.H. and Balmaceda, J.P. (1993) A prospective study of echographic endometrial characteristics and pregnancy rates during hormonal replacement cycles. J. Assist. Reprod. Genet., **3**, 215-219.

412 Shapiro, H., Cowell, C. and Casper, R.F. (1993) The use of vaginal ultrasound for monitoring endometrial preparation in a donor oocyte program. Fertil. Steril., **59**, 1055-1058.

413 Raga, F., Bonilla-Musoles, F., Casan, E.M., Klein, O. and Bonilla, F. (1999) Assessment of endometrial volume by three-dimensional ultrasound prior to embryo transfer: clues to endometrial receptivity. Hum. Reprod., **14**, 2851-2854.

414 Dickey, R.P., Olar, T.T., Taylor, S.N., Curole, D.N. and Harrigill, K. (1993) Relationship of biochemical pregnancy to pre-ovulatory endometrial thickness and pattern in patients undergoing ovulation induction. Hum. Reprod., **2,** 327-330.

415 Dickey, R.P., Olar, T.T., Taylor, S.N., Curole, D.N. and Matulich, E.M. (1993) Relationship of endometrial thickness and pattern to fecundity in ovulation induction cycles: effect of clomiphene citrate alone and with human menopausal gonadotropin. Fertil. Steril., **4,** 756-760.

416 Cohen, B.M., Berry, L., Roethemeyer, V. and Smith, D. (1992) Sonographic assessment of late proliferative phase during ovulation induction. J. Reprod. Med., **37**, 685-690.

417 Check, J.H., Nowroozi, K., Choe, L., Lurie, D. and Dietterich, C. (1993) The effect of endometrial thickness and echo pattern on in vitro fertilization outcome in a donor oocyte embryo transfer cycle. Fertil. Steril., **59**, 72-75.

418 Sher, G., Dodge, S., Maassarani, G., Knutzen, V., Zouves, C. and Feinman, M. (1993) Management of suboptimal sonographic endometrial patterns in patients undergoing in-vitro fertilization and embryo transfer. Hum. Reprod., **8**, 347–349.

419 Hock, D.L., Bohrer, M.K., Ananth, C.V. and Kemmann, E. (1997) Sonographic assessment of endometrial pattern and thickness in patients treated with clomiphene citrate, human menopausal gonadotropins, and intrauterine insemination. Fertil. Steril. **68**, 242-245.

420 Eichler, C., Krampl, E., Reichel, V., Zegermacher, G., Obruca, A., Strohmer, H., Feldner-Busztin, M. and Feichtinger, W. (1993) The relevance of endometrial thickness and echo patterns for the success of in vitro fertilization evaluated in 148 patients. J. Assist. Reprod. Genet., **3**, 223-227.

421 Mardesic, T., Muller, P., Zetova, L., Mikova, M. and Stroufova, A. (1995) Factors affecting the results of in vitro fertilization--III. The effect of the height and properties of the endometrium in the ultrasound image on the probability of implantation. Ceska. Gynekol., **1**, 3-7.

422 Lentz, S. and Lindberg, S. (1990) Ultrasonic evaluation of endometrial growth in women with normal cycles during spontaneous and stimulated cycles. Hum. Reprod., **5**, 377–381.

423 Fanchin, R., Righini, C. and Ayoubi, J.M. (2000) New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization-embryo transfer. Fertil. Steril., **74**, 274-281.

424 Yoshimitsu, K., Nakamura, G. and Nakano, H. (1989) Dating sonographic endometrial images in the normal ovulatory cycle. Int. J. Gynaecol. Obstet., **28**, 33-39.

425 Check, J.H., Dietterich, C. and Lurie, D. (2000) Non-homogeneous hyperechogenic pattern 3 days after embryo transfer is associated with lower pregnancy rates. Hum. Reprod., **15,** 1069-1074.

426 Ohno, Y. and Fujimoto, Y. (1998) Endometrial oestrogen and progesterone receptors and their relationship to sonographic appearance of the endometrium. Hum. Reprod. Update, **5,** 560-564.

427 Bakos, O., Lundkvist, O. and Bergh, T. (1993) Transvaginal sonographic evaluation of endometrial growth and texture in spontaneous ovulatory cycles: a descriptive study. Hum. Reprod., **8,** 799-806.

428 Randall, J.M., Fisk, N.M. and McTavish, A. (1989) Transvaginal ultrasonic assessment of endometrial growth in spontaneous and hyperstimulated menstrual cycles. Br. J. Obstet. Gynaecol., **96**, 954-959.

429 Trounson, A., Leeton, J., Besanko, M., Wood, C. and Conti, A. (1983) Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. Br. Med. J., **12**, 286, 835-838.

430 Toner, J.P., Veeck, L.L., Acosta, A.A. and Muasher, S.J. (1991) Predictive value of pregnancy during original in vitro fertilization cycle on implantation and pregnancy in subsequent cryothaw cycles. Fertil. Steril., **56**, 505-508.

431 Wang, X.J., Ledger, W., Payne, D., Jeffrey, R. and Matthews, C.D. (1994) The contribution of embryo cryopreservation to in-vitro fertilization/gamete intra-fallopian transfer: 8 years experience. Hum. Reprod., **9**,103-109.

432 Tanbo, T., Dale, P.O., Lunde, O., Moe, N. and Abyholm, T. (1995) Obstetric outcome in singleton pregnancies after assisted reproduction. Obstet. Gynecol., **86**, 188-192.

433 Australian In-Vitro Fertilization Collaborative Group (1988) In-vitro fertilization pregnancies in Australia and New Zealand, 1979-1985. Med. J. Aust., **148**, 432-436.

434 Frederick, J.L., Ord, T., Kettel, L.M., Stone, S.C., Balmaceda, J.P. and Asch, R.H. (1995) Successful pregnancy outcome after cryopreservation of all fresh embryos with subsequent transfer into an unstimulated cycle. Fertil. Steril., **64**, 987-990.

435 Selick, C.E., Hofmann, G.E., Albano, C., Horowitz, G.M., Copperman, A.B., Garrisi, G.J. and Navot, D. (1995) Embryo quality and pregnancy potential of fresh compared with frozen embryos--is freezing detrimental to high quality embryos? Hum. Reprod., **10**, 392-395.

436 Hu, Y., Maxson, W.S., Hoffman, D.I., Ory, S.J. and Eager, S. (1999) A comparison of post-thaw results between cryopreserved embryos derived from intracytoplasmic sperm injection and those from conventional IVF. Fertil. Steril., **72**, 1045-1048.

437 Al-Hasani, S., Ludwig, M. and Gagsteiger, F. (1996) Comparison of cryopreservation of supernumerary pronuclear human oocytes obtained after intracytoplasmic sperm injection (ICSI) and after conventional in-vitro fertilization. Hum. Reprod., **11**, 604-647.

438 Ludwig M.Jelkmann, W., Bauer, O. and Diedrich, K. (1999) Prediction of severe ovarian hyperstimulation syndrome by free serum vascular endothetial growth factor concentration on the day of human chorionic gonadotrophin administration. Hum. Reprod., **14**, 2437-2441.

439 Cohen, J., DeVane, G.W., Elsner, C.W., Kort, H.I., Massey, J.B. and Norbury, S.E. (1988) Cryopreserved zygotes and embryos and endocrinologic factors in the replacement cycle. Fertil. Steril., **50**, 61-67.

440 Muasher, S.J., Kruithoff, C., Simonetti, S., Oehninger, S., Acosta, A.A. and Jones, G.S. (1991) Controlled preparation of the endometrium with exogenous steroids for the transfer of frozen-thawed pre embryos in patients with anovulatory or irregular cycles. Hum. Reprod., **6**, 443-445.

441 Testart, J., Lassalle, B., Belaisch-Allart, J., Forman, R., Hazout, A. and Fries, N. (1988) Human embryo freezing. Ann. N.Y. Acad. Sci., **541**, 532-540.

442 Gelety, T. and Surrey, E. (1993) Cryopreservation of embryos and oocytes: an update. Curr. Opin. Obstet. Gynecol., **5**, 606-614.

443 Van der Auwera, I., Meuleman, C. and Koninckx, P.R. (1994) Human menopausal gonadotrophin increases pregnancy rate in comparison with clomiphene citrate during replacement cycles of frozen/thawed pronucleate ova. Hum. Reprod., **9**, 1556-1560.

444 Navot, D., Anderson, T.L., Droesch, K., Scott, R.T., Kreiner, D. and Rosenwaks, Z. (1989) Hormonal manipulation of endometrial maturation. J. Clin. Endocrinol. Metab., **68**, 801–807.

445 Schmidt, C.L., de Ziegler, D., Gagliardi, C.L., Mellon, R.W., Taney, F.H., Kuhar, M.J., Colon, J.M. and Weiss, G. (1989) Transfer of cryopreserved-thawed embryos: the natural cycle versus controlled preparation of the endometrium with gonadotropin-releasing hormone agonist and exogenous estradiol and progesterone (GEEP). Fertil. Steril., **52**, 609-616.

446 Younis, J.S., Simon, A. and Laufer, N. (1996) Endometrial preparation: lessons from oocyte donation. Fertil. Steril., **66**, 873-884.

447 Meldrum, D.R., Wisot, A., Hamilton, F., Gyttay-Yeo, A.L., Marr, B. and Huynh, D. (1989) Artificial agonadism and hormone replacement for oocyte donation. Fertil. Steril., **52**, 509-511.

448 Remohi, J., Gutierrez, A., Cano, F., Ruiz, A., Simon, C. and Pellicer, A. (1995) Long oestradiol replacement in an oocyte donation programme. Hum. Reprod., 10, 1387-1391.

449 Klein J., and Sauer M.V. (2002) Oocyte donation. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol., **3**, 277-291.

450 Lelaidier, C., de Ziegler, D., Gaetano, J., Hazout, A., Fernandez, H. and Frydman, R. (1992) Controlled preparation of the endometrium with exogenous oestradiol and progesterone: a novel regimen not using a gonadotrophin-releasing hormone agonist. Hum. Reprod., **7**, 1353-1356.

451 Simon, A., Hurwitz, A., Zentner, B., Bdolah, Y. and Laufer, N. (1998) Transfer of frozen-thawed embryos in artificially prepared cycles with and without prior gonadotrophin-relasing hormone agonist suppression-a prospective randomized study. Hum. Reprod., **13**, 2712-2717.

452 Simon, A., Hurwitz, A., Pharhat, M., Revel, A., Zentner, B.S. and Laufer, N. (1999) A flexible protocol for artificial preparation of the endometrium without prior gonadotropin-releasing hormone agonist suppression in women with functioning ovaries undergoing frozen-thawed embryo transfer cycles. Fertil. Steril., **71**, 609–613.

453 Queenan, Jr. J.T., Ramey, J.W., Seltman, H.J., Eure, L. and Veeck, L.L. (1997) Transfer of cryopreserved-thawed pre-embryos in a cycle using exogenous steroids without prior gonadotrophin-releasing hormone agonist suppression yields favourable pregnancy results. Hum. Reprod., **12**, 1176-1180.

454 Pattinson, H.A., Greene, C.A., Fleetham, J. and Anderson-Sykes, S.J. (1992) Exogenous control of the cycle simplifies thawed embryo transfer and results in a pregnancy rate similar to that for natural cycle. Fertil. Steril., **58**, 627-629.

455 Serhal, P. and Craft, I. (1987) Ovum donation- a simplified approach. Fertil. Steril., **48**, 265-269.

456 Leeton, J., Rogers, P, King, C. and Healy, D. (1991) A comparison of pregnancy rates for 131 donor oocyte transfers using either a sequential or fixed regime of steroid replacement. Hum. Reprod., **6**, 299-301.

457 Zegers-Hochschild, F., Fernandez, E. and Fabres, C. (1992) Pregnancy rate in an oocyte donation program. J. Assist. Reprod. Genet., **9**, 350-352.

458 Kogosowski, A., Yovel, I., Lessing, J.B., Amit, A., Barak, Y., David, M.P. (1990) The establishment of an ovum donation program using a simple fixed dose estrogen-progesterone replacement regimen. J. In Vitro Fert. Embryo Transf., **7**, 244-248

459 Navot, D., laufer, N., Kopolovic, J., Rabinovwitz, R., Birkenfeld, A. and Lewin, A. (1986) Artificially induced cycles and establishment of pregnancies in the absence of ovaries. N. Engl. J. Med., **314**, 806-811.

460 Younis, J.S., Mordel, N., Lewin, A., Simon, A., Schenker, J.G. and Laufer, N. (1992) Artificial endometrial preparation for oocyte donation: the effect of estrogen stimulation on clinical outcome. J. Assist. Reprod. Genet., **9**, 222-227.

461 Lutjen, P., Trounson, A. and Leeton, J. (1984) The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. Nature, **307**, 174-175.

462 Rosenwaks, Z. (1987) Donor eggs: their application in modern reproductive technologies. Fertil. Steril., **47**, 895-909.

463 Lutjen, P.J., Findlay, J.K., Trounson, A.O., Leeton, J.F. and Chan, L.K. (1986) Effect on plasma gonadotropins of cyclic steroid replacement in women with premature ovarian failure. J. Clin. Endocrinol. Metab., **62**, 419-423.

464 Asch, R.H., Balmaceda, J.P., Ord, T., Borrero, C., Cefalu, E. and Gastaldi, C. (1988) Oocyte donation and gamete intrafallopian transfer in premature ovarian failure. Fertil. Steril., **49**, 263-267.

465 Ben-Nun, I. and Shulman, A. (1997) Induction of artificial endometrial cycles with s.c. oestrogen implants and injectable progesterone in in-vitro fertilization treatment with donated oocytes: a preliminary report. Hum. Reprod., **12**, 2267-2270.

466 Dmowski, W.P., Michalowska, J., Rana, N., Friberg, J., McGill-Johnson, E. and DeOrio, L. (1997) Subcutaneous estradiol pellets for endometrial preparation in donor oocyte recipients with a poor endometrial response. J. Assist. Reprod. Genet., **14**, 139-144.

467 Stovall, D.W., Van Voorhis, B.J., Mattingly, K.L., Sparks, A.E., Chapler, F.K. and Syrop, C.H. (1996) The effectiveness of sublingual progesterone administration during cryopreserved embryo transfer cycles: results of a matched follow-up study. Fertil. Steril., **65**, 986-991.

468 Tavaniotou, A., Smitz, J., Bourgain, C. and Devroey, P. (2000) Comparison between different routes of progesterone administration as luteal phase support in infertility treatments. Hum. Reprod. Update, **6**, 139-148.

469 Prato, L.D., Borini, A., Cattoli, M., Bonu, M.A., Sciajno, R. and Flamigni, C. (2002) Endometrial preparation for frozen-thawed embryo transfer with or without pretreatment with gonadotropin-releasing hormone agonist Fertil. Steril., **5**, 956-960.

470 Michalas, S., Loutradis, D., Drakakis, P., Kallianidis, K., Milingos, S., Deligeoroglou, E. and Aravantinos, D. (1996) A flexible protocol for the induction of recipient endometrial cycles in an oocyte donation programme. Hum. Reprod., **11**, 1063-1066.

471 Remohi, J., Vidal, A. and Pellicer, A. (1993) Oocyte donation in low responders to conventional ovarian stimulation for in vitro fertilization. Fertil. Steril., **59**, 1208–1215.

472 Krasnow, J.S., Berga, S.L., Guzick, D.S,, Zeleznik, A.J. and Yeo, K,T. (1996) Vascular permeability factor and vascular endothelial growth factor in ovarian hyperstimulation syndrome: a preliminary report. Fertil. Steril., **3,** 552-555.

473 Prapas, Y., Prapas, N., Jones, E.E., Duleba, A.J., Olive, D.L., Chatziparasidou, A. and Vlassis, G. (1998) The window for embryo transfer in oocyte donation cycles depends on the duration of progesterone therapy. Hum. Reprod., **13**, 720-723.

474 Navot, D., Bergh, P.A., Williams, M., Garrisi, G.J., Guzman, I. and Sandler, B. (1991) An insight into early reproductive processes through the in vivo model of ovum donation. J. Clin. Endocrinol. Metab., **72**, 408–414.

475 Younis, J.S., Mordel, N., Ligovetzky, G., Lewin, A., Schenker, J.G. and Laufer, N. (1991) The effect of a prolonged artificial follicular phase on endometrial development in an oocyte donation program. J. In Vitro Fert. Embryo Transf., **8**, 84-88.

476 Yaron, Y., Amit, A., Mani, A., Yovel, I., Kogosowski, A., Peyser, M.R., David, M.P. and Lessing, J.B. (1995) Uterine preparation with estrogen for oocyte donation: assessing the effect of treatment duration on pregnancy rates. Fertil. Steril., **63**, 1284-1286.

477 Borini, A., Dal Prato, L., Bianchi, L., Violini, F., Cattoli, M. and Flamigni, C. (2001) Effect of duration of estradiol replacement on the outcome of oocyte donation. J. Assist. Reprod. Genet., **18**, 187-192.

478 Sathanandan, M., Macnamee, M.C., Rainsbury, P., Wick, K., Brinsden, P. and Edwards, R.G. (1991) Replacement of frozen-thawed embryos in artificial and natural cycles: a prospective semi-randomized study. Hum. Reprod., **6**, 685-687.

479 Queenan, J.T., Jr, Veeck, L.L., Seltman, H.J. and Muasher, S.J. (1994) Transfer of cryopreserved-thawed pre-embryos in a natural cycle or a programmed cycle with exogenous hormonal replacement yields similar pregnancy results. Fertil. Steril., **62**, 545-550.

480 Dor, J., Rudak, E., Davidson, A., Levran, D., Ben-Rafael, Z. and Mashiach, S. (1991) Endocrine and biological factors influencing implantation of human embryos following cryopreservation. Gynecol. Endocrinol., **5**, 203-211.

481 Imthurn, B., Macas, E., Rosselli, M. and Keller, P.J. (1996) Effect of a programmed short-term stimulation protocol on the replacement of cryopreserved embryos. J. Assist. Reprod. Genet.,**13**, 709-712.

482 Tanos, V., Friedler, S., Zajicek, G., Neiger, M., Lewin, A. and Schenker, J.G. (1996) The impact of endometrial preparation on implantation following cryopreserved-thawed-embryo transfer. Gynecol. Obstet. Invest., **41**, 227-231.

483 Kondo, I., Suganuma, N., Ando, T., Asada, Y., Furuhashi, M. and Tomoda, Y. (1996) Clinical factors for successful cryopreserved-thawed embryo transfer. J. Assist. Reprod. Genet., **13**, 201-206.

484 Wang, J.X., Yap, Y.Y. and Matthews C.D. (2001) Frozen–thawed embryo transfer: influence of clinical factors on implantation rate and risk of multiple conception Hum. Reprod., **11**, 2316-2319.

485 Loh, S.K. and Leong, N.K. (1999) Factors affecting success in an embryo cryopreservation programme. Ann. Acad. Med. Singapore, **28**, 260-265.

486 Cameron, I.T., Rogers, P.A., Caro, C., Harman, J., Healy, D.L., Leeton, J.F. (1989) Oocyte donation: a review. Br. J. Obstet. Gynaecol., **96**, 893-899.

487 de Ziegler, D., Frydman, R. (1990) Different implantation rates after transfers of cryopreserved embryos originating from donated oocytes or from regular in vitro fertilization. Fertil. Steril., **54**, 682-688.

488 Schalkoff ME, Oskowitz SP and Powers RD. (1993) A multifactorial analysis of the pregnancy outcome in a successful embryo cryopreservation program. Fertil. Steril. **59**,1070-1074.

489 Battaglia, C., Artini, P.G. and D'Ambrogio G (1994) Uterine and ovarian blood flow measurement. Does the full bladder modify the flow resistance? Acta. Obstet. Gynecol. Scand., **73**, 716-718.

490 Miles, R.D., Menke, J.A., Bashiru, M. and Colliver, J.A. (1987) Relationships of five Doppler measures with flow in an in vitro model and clinical findings in newborn infants. J. Ultrasound Med., **6**, 597–599.

491 Webb, N.J., Bottomley, M.J., Watson, C.J. and Brenchley, P.E. (1998) Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. Clin. Sci. (Lond)., **4,** 395-404.

492 Barak, V., Mordel, N. and Zajicek, G. (1992) The correlation between interleukin 2 and soluble interleukin 2 receptors to oestradiol, progesterone and testosterone levels in periovulatory follicles of in-vitro fertilization patients. Hum. Reprod., **7**, 926-929

493 Mendoza, C., Cremades, N. and Ruiz-Requena, E. (1999) Relationship between fertilization results after intracytoplasmic sperm injection, and intrafollicular steroid, pituitary hormone and cytokine concentrations. Hum. Reprod., **14**, 628-635.

494 Strandell, A. and Lindhard, A. (2002) Why does hydrosalpinx reduce fertility? The importance of hydrosalpinx fluid. Hum. Reprod., **5**, 1141-1145.

495 Kably Ambe, A., Barron Vallejo, J., Tapia Lizarraga, R.C. and Krivitsky, S.K. (2000) Effect of blood concentrations of preovulatory estradiol on the quality of eggs and pre-embryos in patients treated with fertilization in vitro. Ginecol. Obstet Mex., **68**, 435-441.

496 Ferraretti, A.P., Gianaroli, L., Magli, M.C., Bafaro, G. and Colacurci, N. (2000) Female poor responders. Mol. Cell Endocrinol., **1-2**, 59-66.

497 Karande, V.C., Jones, G.S., Veeck, L.L. and Muasher S.J. (1990) High-dose follicle-stimulating hormone stimulation at the onset of the menstrual cycle does not improve the in vitro fertilization outcome in low-responder patients. Fertil. Steril., **3**,:486-489.

498 Van Hooff, M.H., Alberda, A.T., Huisman, G.J., Zeilmaker, G.H. and Leerentveld, R.A. (1993). Doubling the human menopausal gonadotrophin dose in the course of an in-vitro fertilization treatment cycle in low responders: a randomized study. Hum. Reprod., **3**, 369-373.

499 Out, H.J., Braat, D.D., Lintsen, B.M., Gurgan, T., Bukulmez, O., Gokmen, O., Keles, G., Caballero, P., Gonzalez, J.M., Fabregues, F., Balasch, J. and Roulier, R. (2000) Increasing the daily dose of recombinant follicle stimulating hormone (Puregon) does not compensate for the age-related decline in retrievable oocytes after ovarian stimulation. Hum. Reprod., **1**, 29-35.

500 Out, H.J., David, I., Ron-El, R., Friedler, S., Shalev, E., Geslevich, J., Dor, J., Shulman, A., Ben-Rafael, Z., Fisch, B. and Dirnfeld, M. (2001) A randomized, double-blind clinical trial using fixed daily doses of 100 or 200 IU of recombinant FSH in ICSI cycles. Hum. Reprod., **6**, 1104-1109.

501 Harrison, R.F., Jacob, S., Spillane, H., Mallon E. and Hennelly, B. (2001) A prospective randomized clinical trial of differing starter doses of recombinant follicle-stimulating hormone (follitropin-beta) for first time in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil. Steril., **75**, 23–31.

502 Land, J.A., Yarmolinskaya, M.I., Dumoulin, J.C.M. and Evans, J.H. (1996) High-dose human menopausal gonadotrophin stimulation in poor responders does not improve the in vitro fertilization outcome. Fertil. Steril., **65**, 961-965.

503 Bertrand, E., Van den Bergh, M. and Englert, Y. (1996) Clinical parameters influencing human zona pellucida thickness. Fertil. Steril., **66**, 408-411.

504 Franks, S., Roberts, R. and Hardy, K. (2003) Gonadotrophin regimens and oocyte quality in women with polycystic ovaries. Reprod. Biomed. Online., **2**, 181-184

514 Wheeler, C.A., Cole, B.F., Frishman, G.N., Seifer, D.B., Lovegreen, S.B. and Hackett, R.J. (1998) Predicting probabilities of pregnancy and multiple gestation from in vitro fertilization: a new model. Obstet. Gynecol. **91**, 696-700.

515 Hunault, C.C., Eijkemans, M.J., Pieters, M.H., Te Velde, E.R., Habbema, J.D., Fauser, B.C. and Macklon, N.S. (2002) A prediction model for selecting patients undergoing in vitro fertilization for elective single embryo transfer. Fertil. Steril., **4,** 725-732.

516 Gerris, J., De Neubourg, D. and Van Royen, E. (2000) Prevention of twin pregnancy after IVF/ICSI based on strict embryo criteria: final results of a prospective randomized clinical trial including neonatal data. Hum. Reprod., **15**, Abstract Book of the sixteenth Annual Meeting of ESHRE, 27.

517 Coetsier, T. and Dhont, M. (1998) Avoiding multiple pregnancies in in-vitro fertilization: who's afraid of single embryo transfer? Hum. Reprod., 13, 2663-2664.

518 Cohen, J. (1998) Embryo replacement technology. 31st Annual Postgraduate Programme of the American Society for Reproductive Medicine, San Francisco, California., 177-184.

519 Goudas, V.T., Hammitt, D.G., Damario, M.A., Session, D.R., Singh, A.P. and Dumesic, D.A. (1998) Blood on the embryo transfer catheter is associated with decreased rates of embryo implantation and clinical pregnancy with the use of in vitro fertilization-embryo transfer. Fertil. Steril., **5**, 878-882.

520 Sharif, K., Afnan, M. and Lenton, W. (1995) Mock embryo transfer with a full bladder immediately before the real transfer for in-vitro fertilization treatment: the Birmingham experience of 113 cases. Hum, Reprod., **7**, 1715-1718.

521 Mansour, R., Aboulghar, M. and Serour, G. (1990) Dummy embryo transfer: a technique that minimizes the problems of embryo transfer and improves the pregnancy rate in human in vitro fertilization. Fertil. Steril., **4**, 678-681.

522 Tur-Kaspa, I., Yuval, Y., Bider, D., Levron, J., Shulman, A. and Dor, J.(1998) Difficult or repeated sequential embryo transfers do not adversely affect in-vitro fertilization pregnancy rates or outcome. Hum, Reprod., **9**, 2452-2455.

523 Strickler, R.C., Christianson, C., Crane, J.P., Curato, A., Knight, A.B. and Yang, V. (1985) Ultrasound guidance for human embryo transfer. Fertil. Steril., **1**, 54-61.

524 Coroleu, B., Carreras, O., Veiga, A., Martell, A., Martinez, F., Belil, I., Hereter, L. and Barri, P.N. (2000) Embryo transfer under ultrasound guidance improves pregnancy rates after in-vitro fertilization. Hum. Reprod., **3**, 616-620.

525 Battaglia, C., Larocca, E. and Lanzani, A. (1990) Doppler ultrasound studies of the uterine arteries in spontaneous and IVF stimulated ovarian cycles. Gynecol. Endocrinol., **4**, 245-250.

526 Sher, G. and Fisch, J.D. (2000) Vaginal sildenafil (Viagra): a preliminary report of a novel method to improve artery blood flow and endometrial development in patients undergoing IVF. Hum. Reprod., **15**, 806–809.

527 Ohl, J., Lefebvre-Maunoury, C., Wittemer, C., Nisand, G., Laurent, M.C. and Hoffmann, P. (2002) Nitric oxide donors for patients undergoing IVF. A prospective, double-blind, randomized, placebo-controlled trial. Hum. Reprod., **10**, 2615-2620.

528 Shaker, A.G., Fleming, R., Jamieson, M.E., Yates, R.W. and Coutts, J.R. (1993) Assessments of embryo transfer after in-vitro fertilization: effects of glyceryl trinitrate. Hum. Reprod., **8**, 1426-1428.

529 Reilly, I.A. and FitzGerald, G.A. (1987) Inhibition of thromboxane formation in vivo and ex vivo: implications for therapy with platelet inhibitory drugs. Blood, **69**, 180-186.

530 Hamberg, M. and Samuelsson, B. (1974) Prostaglandin endoperoxides, novel transformations of arachidonic acid in human platelets. Proc. Natl. Acad. Sci. USA, **71**,3400-3404.

531 Schror K. (1997) Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. Semin. Thromb. Hemost., **23**, 349-356.

532 Elwood P.C. and Stillings M.R. (2000) Use of aspirin in cardiovascular prophylaxis. Cardiovasc. J. S. Afr., **11**,155-160.

533 European Action on Secondary Prevention by Intervention to Reduce Events. (2001) EUROASPIRE I and II Group Clinical reality of coronary prevention guidelines: a comparison of EUROASPIRE I and II in nine countries. EUROASPIRE I and II Group. Lancet, **31**, 995-1001.

534 Rai, R., Backos, M. and Baxter, N. (2000) Recurrent miscarriage-an aspirin a day? Hum. Reprod., **15**, 2220-2223.

535 Moncada, S., Gryglewski, R. and Bunting, S. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature, **263**,663-665.

536 Blumenfeld, Z., Weiner, Z. and Lorber, M. (1991) Anticardiolipin antibodies in patients with recurrent pregnancy wastage: treatment and uterine blood flow. Obstet. Gynecol., **78**, 584-589.

537 Levin, J.H., Stanczyk, F.Z. and Lobo, R.A. (1992) Estradiol stimulates the secretion of prostacyclin and thromboxane from endometrial stromal cells in culture. Fertil. Steril., **58**, 530-536.

538 Giorlandino, C., Gleicher, N. and Nanni, C. (1987) The sonographic picture of endometrium in spontaneous and induced cycles. Fertil. Steril., **47**, 508-511.

539 Check, J.H., Lurie, D., Dietterich, C., Callan, C. and Baker, A. (1993) Adverse effect of a homogenous hyperechogenic endometrial sonographic pattern, despite adequate endometrial thickness on pregnancy rates following in vitro fertilization. Hum. Reprod., **8**, 1293-1296.

540 De Geyter, C., Schmitter, M., De Geyter, M., Nieschlag, E., Holzgreve, W. and Schneider, H.P. (2000) Prospective evaluation of the ultrasound appearance of the endometrium in a cohort of 1,186 infertile women. Fertil. Steril., **1**,106-113.

541 Yaman, C., Ebner, T., Sommergruber, M., Pölz, W. and Tews, G. (2000) Role of three-dimensional ultrasonographic measurement of endometrium volume as a predictor of pregnancy outcome in an IVF-ET program: a preliminary study. Fertil. Steril., **4**, 797-801.

542 Rinaldi, L., Lisi, F., Floccari, A., Lisi, R., Pepe, G. and Fishel, S. (1996) Endometrial thickness as a predictor of pregnancy after in-vitro fertilization but not after intracytoplasmic sperm injection. Hum. Reprod., **7**, 1538-1541.

543 Weissman, A., Gotlieb, L. and Casper, R.F. (1999) The detrimental effect of increased endometrial thickness on implantation and pregnancy rates and outcome in an in vitro fertilization program. Fertil. Steril., **71**, 147-149.

544 Hornig, C. And Weich, H.A. (1999) Soluble VEGF receptors. Angiogenesis, **3**, 33-39.

545 Sharkey, A.M., Day, K., McPherson, A., Malik, S., Licence, D., Smith, S.K. and Charnock-Jones, D.S. (2000) Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. J. Clin. Endocrinol. Metab., **1**, 402-409.

546 Ahmad, S. and Ahmed, A. (2005) Antiangiogenic effect of soluble vascular endothelial growth factor receptor-1 in placental angiogenesis. Endothelium, **1-2**, 89-95.

547 Barroso, G., Barrionuevo, M., Rao, P., Graham, L., Danforth, D., Huey, S., Abuhamad, A. and Oehninger, S. (1999) Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil. Steril., **6**, 1024-1026.

548 Tokuyama, O., Nakamura, Y., Muso, A., Fujino, Y., Ishiko, O. and Ogita, S. (2002) Vascular endothelial growth factor concentrations in follicular fluid obtained from IVF-ET patients: a comparison of hMG, clomiphene citrate, and natural cycle. J. Assist. Reprod. Genet., **1,** 19-23.

549 Karlstrom, P.O., Bergh, T., Forsberg, A.S., Sandkvist, U. and Wikland, M. (1997) Prognostic factors for the success rate of embryo freezing. Hum. Reprod., **12**, 1263-1266.

550 Garcia, E., Bouchard, P., De Brux, J., Berdah, J., Frydman, R. and Schaison, G. (1988) Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. J. Clin. Endocrinol. Metab., **67**, 80–87.

551 Konc, J., Kanyo, K. and Cseh, S. (2005) Clinical experiences of ICSI-ET thawing cycles with embryos cryopreserved at different developmental stages. J. Assist. Reprod. Genet., **5**, 185-190.

552 Marrs, R.P., Greene, J. and Stone, B.A. (2004) Potential factors affecting embryo survival and clinical outcome with cryopreserved pronuclear human embryos. Am. J. Obstet. Gynecol., **6**, 1766-1771.

553 Check, M.L., Check, J.H., Summers-Chase, D., Swenson, K. and Yuan, W. (2001) Pregnancy/implantation rates as related to age following transfer of frozen embryos produced by ICSI. Arch. Androl., **3,** 161-165.

554 Salumets, A., Tuuri, T., Makinen, S., Vilska, S., Husu, L., Tainio, R. and Suikkari AM. (2003) Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen-thawed embryo transfer. Hum. Reprod., **9**, 1890-1895.

555 Zhang, L., Rees, M.C. and Bicknell, R. (1995) The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges J. Cell. Sci.., **108**, 323-331.

556 Hyder, S.M., Nawaz, Z., Chiappetta, C. and Stancel, G.M. (2000) Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res., **12**, 3183-3190.

557 Hazzard, T,M, Molskness, T.A., Chaffin,C.L. and Stouffer, R.L. (1999) Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granutosa cells during the periovulatory interval. Mol. Hum. Reprod., **5**, 1115-1121.

558 Hazzard, T.M. and Stouffer, R.L. (2000) Angiogenesis in ovarian follicular and luteal development. In Arulkumaran S, ed. Clinical Obstetrics & Gynaecology. Angiogenesis in the Female Reproductive Tract. London: Bailliere Tindall., 883-900.

559 Torry, D.S., Holt, V.J., Keenan, J.A., Harris, G., Caudle, M.R. and Torry, R.J.(1996) Vascular endothelial growth factor expression in cycling human endometrium. Fertil. Steril., **1,** 72-80.

560 Shifren, J.L., Tseng, J.F., Zaloudek, C.J., Ryan, I.P., Meng, Y.G., Ferrara, N., Jaffe, R.B. and Taylor, R.N. (1996) Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J. Clin. Endocrinol. Metab., **81,** 3112-3118.

561 Clark, D.F., Smith, S.K., Sharkey, A.M. and Charnock-Jones, D.S. (1996) Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. Hum. Reprod., **11**, 1090-1098.

562 Sharkey, A.M., Cooper, J.C, Balmforth J.R, McLaren, J., Clark, D.E., Charnock-Jones, D.S., Morris, N.H. and Smith, S.K. (1996) Maternal plasma levels of vascular endothelial growth factor in normotensive pregnancies and in pregnancies complicated by pre-eclampsia. Eur J Clin Invest., **12**, 1182-1185.

563 Banz, C., Katalinic, A., Al-Hasani, S., Seelig, A.S., Weiss, J.M., Diedrich, K. and Ludwig, M. (2002) Preparation of cycles for cryopreservation transfers using estradiol patches and Crinone® 8% vaginal gel is effective and does not need any monitoring Eur. J. Obstet. Gynecol. Reprod. Biol., **1**, 43-47.

564 Turnbull, L.W., Lesny, P. and Killick, S.R. (1995) Assessment of uterine receptivity prior to embryo transfer: a review of currently available imaging modalities. Hum. Reprod. Update, **5**, 505-514.

10 List of Tables and Figures